TRANSPORTER GENE EXPRESSION IN RAT LACTATING MAMMARY EPITHELIAL CELLS & PRIMARY ORGANOID CULTURES USING QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QRT-PCR)

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ABSTRACT

Transporters dynamically expressed at the mammary gland transport critical nutrients into the breast milk of nursing mothers to meet the nutritional demands of the suckling infant. However, xenobiotics may interact with these transporters to potentially alter the nutrient composition of milk and compromise neonatal nutrition. The aim of the present study was to quantitatively evaluate the constitutive expression of various nutrient transporters in whole mammary gland tissue and mammary epithelial organoids (MEO) isolated from female Sprague-Dawley rats at various stages of pregnancy, lactation, and involution. Furthermore, the study's aim was to determine if appropriately cultured mammary epithelial organoids (MEO) maintain *in vivo* transporter expression to lay down critical groundwork for the development of an *in vitro* screening tool assessing xenobiotic-nutrient transporter interactions. The following transporters were evaluated using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR): multidrug resistance protein (Mdr) 1a, 1b; multidrug resistance-like protein (Mrp) 1; organic cation transporter (Oct) 1; organic cation/carnitine transporter (Octn) 1, 2, and 3; concentrative nucleoside transporter (Cnt) 1, 2, and 3; equilibrative nucleoside transporter (Ent) 1, 2, and 3; nucleobase transporter (Ncbt) 1 and 2; oligopeptide transporter (Pept) 1 and 2; methotrexate carrier (Mtx) 1; divalent metal transporter (Dmt) 1; and the milk protein β-casein. Transporter expression patterns in MEO differed from whole tissue for β-actin, Mdr1a, Mdr1b, Oct1, Octn3, Ent3, Cnt1, Cnt3, Ncbt1, Pept2, Mtx1, and β-casein. This brings into question whether whole mammary gland tissue is truly appropriate for an understanding of transporter expression in the mammary epithelium. Nevertheless, four general transporter expression patterns emerged in isolated MEO: decline throughout lactation (Mdr1a, Mdr1b, Mrp1 & Dmt1), increase throughout lactation (Cnt1 & Octn3), increase in early lactation (Oct1, Octn2, Ent1, Cnt2, Cnt3, Pept2 & Mtx1) and constant expression throughout lactation (Octn1, Ent2, Ent3, Ncbt1, Ncbt2 & Pept1). These expression patterns will provide insight into the critical windows of nutrient delivery to the breast milk to provide adequate nutritional stimuli to the suckling infant. Furthermore, MEO cultured in an extracellular matrix-rich environment maintained transporter expression at the mRNA level, which underscores the potential of the primary MEO *in vitro* model system as a screening tool

for xenobiotic-transporter interactions at the mammary gland. Transporter expression patterns in MEO were unique for each transporter evaluated. This information accompanied by an *in vitro* screening tool may allow for predictions of xenobiotic interference with breast milk composition to help safeguard infant health.

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For Mom, Dad, Matt, & Rudy

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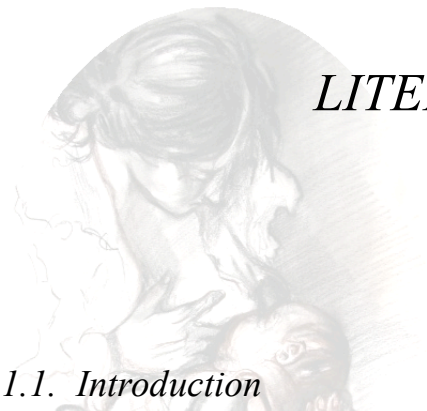
ABBREVIATION LIST

Abbreviation or Symbol	
AAP	American Academy of Pediatrics
ABC	ATP-binding cassette superfamily of transport proteins
ACV	Acyclovir
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AZT	Zidovudine
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BMI	Body Mass Index
Bsep	Bile salt excretory protein
cADP-R	Cyclic-Adenosine diphosphate-ribose
cAMP	Cyclic-Adenosine monophosphate
CCAC	Canadian Council for Animal Care
Cnt	Concentrative nucleoside transporter family
CSF	Cerebrospinal fluid
C_T	Threshold cycle
CYP	Cytochrome P450
Type I DM	Insulin-dependent diabetes mellitus
Type II DM	Non-insulin-dependent diabetes mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
Dmt1	Divalent metal transporter 1
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarm matrix
EI	Exposure index
Ent	Equilibrative nucleoside transporter family
FAD	Flavin adenine dinucleotide

FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCV	Gancyclovir
GI	Gastrointestinal
GLUT	Glucose transporter
GTP	Guanine triphosphate
HDL	High-density lipoprotein
Ibat	Ilial bile acid transporter
IGF-1	Insulin-like growth factor 1
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low-density lipoprotein
Mdr	Multidrug resistance protein (P-glycoprotein)
MEO	Mammary epithelial organoids
mRNA	Messenger ribose nucleic acid
Mrp	Multidrug resistance-like protein
Mtx1	Methotrexate carrier 1
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Ncbt	Nucleobase transporter family
Oat	Organic anion transporter
Oatp	Organic anion transporting polypeptide
Oct1	Organic cation transporter family – Member 1
Octn	Organic cation/carnitine transporter family
PBS	Phosphate buffered saline
Pept	Oligopeptide transporter family
P_i	Inorganic phosphate
PTHrP	Parathyroid hormone-related protein
QRT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction
RNA	Ribose nucleic acid

SLC	Solute carrier superfamily of transport proteins
Stcp	Sodium taurocholate cotransporting polypeptide
SVCT	Sodium-dependent vitamin C transporter
Tst	Testis-specific transporter
WHO	World Health Organization
Znt	Zinc transporter



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LITERATURE REVIEW

1.1. Introduction

Millions of years of evolution have transformed the mammary gland from an accidentally hypertrophied gland, whose secretions lacked any basic nutritional value into an extremely specified and efficient organ which now provides all mammalian young with a highly regulated nutrient-rich secretion[1, 2]. However, the protein-mediated pathways that regulate the transport of critical macro- and micronutrients into the milk[3, 4] and consequently govern the specificity of milk composition have received limited attention. Yet without a fully developed knowledge of many of the critical processes responsible for the regulation of the nutrient composition of maternal milk, breastfeeding is nevertheless being promoted worldwide because of the proposed health benefits over that of infant formula[5-7]. In developed and underdeveloped countries alike, breastfeeding is promoted as the best form of infant feeding. However, in underdeveloped countries plagued with poor nutrition and/or environmental toxins, and developed countries with similar environmental risks and copious use of pharmaceuticals, breastfeeding is encouraged with very few contraindications[8-14]. What is not well known is whether environmental factors, such as environmental toxins or exogenous xenobiotic use can negatively influence the composition of breast milk. Through breastfeeding, mothers in a nutrient scarce environment pass a poor-nutrition phenotype to her progeny leading to a permanent

reorganization of the neonatal system[15-20]. In addition, if drugs/toxins are involved, the knowledge of drug/toxin levels in the breast milk is inadequate, despite contemporary thought to the contrary, for risk/benefit assessments when mothers who wish to breastfeed require pharmacotherapeutic management for chronic disease conditions. This is because many of these clinically relevant xenobiotics, which are not contraindicated during breastfeeding, are substrates for nutrient-specific transporters. An interaction between a xenobiotic and a nutrient transporter may compromise the nutrient composition of maternal milk leading to an altered neonate phenotype. It is not known under what circumstances these exposures to xenobiotics will compromise milk composition, and pose a significant nutritional risk to the nursing neonate. A comprehensive investigation of the temporal expression of mammary gland transport proteins will aid in assessing when a potential xenobiotic-transporter interaction may occur at the lactating mammary gland. Furthermore, an *in vitro* screening tool will help to screen such interactions, providing a critical assessment to help safeguard both mother and infant health.

1.2. Background

1.2.1. Breastfeeding – Evolutionary Perspectives

> *"Is it conceivable that the young of any animal was ever saved from destruction by accidentally sucking a drop of scarcely nutritious fluid from an accidentally hypertrophied cutaneous gland of its mother?"*
>
> - *St. George Mivart* [21]

The mammary gland is one of the most remarkable developmental systems in human and animal physiology, and despite the fact that the mammary gland and lactation are the sole defining characteristics of all mammals, it's origin and evolution is largely a mystery. Postulated to have evolved from a common ancestor as early as the Jurassic period[1], this gland may have evolved from a holocrine sebaceous gland, an exocrine sweat gland, or an apocrine scent gland[1, 2]. However, regardless of the origin

of this gland, the nutrient-rich secretions that it now provides serve as a feeding-station for the suckling young of all mammals.

Our evolved reproductive strategy has developed in the wake of our primate ancestors. We compromise large litter sizes for a longer gestation period and considerably longer lactation periods. The benefits are speculatory, but are best summed up by Malcolm Peaker[2], who speculates that the long period of parental control had a very distinct evolutionary advantage: Long gestational periods and birth through the pelvic route limited infant size to an extent that they were developmentally immature, specifically in neural development. Neural plasticity allowed for a learned adaptation to the environment and has therefore demanded prolonged parental care beyond that of lactation in higher order animals such as primates and Homo sapiens[2]. This "prolonged care" hypothesis has allowed us to gleam information about the mother-neonate interaction during lactation in that it may not be just a nutrient-providing interaction but may play a significant role in ensuring the developing offspring are programmed to be the most reproductively fit (for survival) as possible to thrive by accurately reflecting the immediate environment (i.e. nutritional resources)[2].

The nutrient messages in maternal milk may accurately reflect the nature of the immediate maternal environment – a signal to the neonate of what is to be expected after weaning – to enhance the success of offspring survival[2]. Or milk may merely provide adequate nutrients to immature animals who are unable to thrive on their own to avoid destruction[2]. Regardless of the proposed role of the mammary gland in lactation and infant feeding/signaling, lactation is without a doubt an extremely critical and important stage in mammalian development and survival. Our current knowledge base of mammary gland function precludes an adequate understanding of the roles of this critical organ in the development and survival of our young.

1.2.1.1. Early Life Nutrition and its Consequences in Later Life

The majority of mammals, specifically the higher order mammals such as humans and primates, have a distinct evolutionary advantage over many other species. Mammals are born very neural-plastic and metabolically immature and can, therefore, rapidly and permanently adapt to the external environment in a manner best suited for survival[2]. Mammals all evolved from a common ancestor millions of years ago;

however, we all exist in very different and ever-changing environments and we readily and sometimes unknowingly adapt to these subtle environmental insults as they transpire. Programmed information must be passed on to our young to ensure their survival[1, 2]. The mammary gland is the sole nutrient-providing source for mammalian neonates and may act as one of the many signaling mechanisms to indicate to the offspring what to expect in the current environment, at least with respect to readily available food sources. If food sources are in abundance, litters are allowed to grow and feast with no limits. However, if food sources become immediately scarce, litters must adapt to an environment low in resources. This phenomenon was initially described over 40 years ago when rat litters from two mothers were manipulated[16, 17]. One dam received more pups than she could handle, and the other dam received only a very small litter. Upon weaning, animals from the large litter group with limited milk availability were significantly smaller than those from the small litter group where milk was in abundance. Furthermore, as animals were introduced to normal rat chow, the smaller animals from the large litter group continued to remain small in size relative to the small littered animals indicating a permanent reorganization of metabolism in the smaller animals[16, 17]. Although genetic factors determine the genotype of a species, other mechanisms in addition to genetic factors can play a role in determining the phenotype. These non-genetic, or epigenetic mechanisms, play a significant role determining the organization of every physiological system[18]. In fact, the alterations in metabolism from such epigenetic mechanisms are well established, and can cause significant long-term alterations in metabolism[18].

Since the mammary gland is the sole nutrient-providing organ for mammals in the initial stages of life and has evolved numerous adaptive mechanisms to permanently organize the physiology of the offspring to ensure their immediate survival to reproduce, we must begin to appreciate the dynamics of this system and question the consequences that these dynamics may have on the offspring in later life.

1.2.1.2. Maternal Adaptations to Mammary Gland Differentiation and Milk Secretion

The status of the mammary gland is dependent on reproductive state and the demands of the nursing young for milk. The mammary gland is a hormonally regulated system dependent on endocrine control from reproductive hormones (estrogen,

progesterone, placental lactogen, prolactin, and oxytocin), metabolic hormones (growth hormones, corticosteroids, thyroid hormone, and insulin), and mammary hormones (growth hormone, prolactin, leptin, and PTHrP)[22]. The development of the mammary gland from virgin to a fully functional, milk-secreting organ is due to many hormonal interventions and has been recently reviewed[22-24]. My thesis will only cover the basics with respect to mammary gland function during pregnancy and lactation and will not review the mammary gland from embryonic development to puberty.

The onset of pregnancy stimulates a significant remodeling process within the mammary gland with continuous changes leading up to lactogenesis and persisting throughout the entire lactation period; lactogenesis phase I and II, respectively (Figure 1.1). Lactogenesis phase I occurs during mid-pregnancy with the proliferation and differentiation of the terminal end buds and the expression of most (but not all) genes responsible for milk production in the alveolar cells[22]. Lactogenesis phase I is controlled primarily by the reproductive hormones progesterone, prolactin, placental lactogen, and growth hormone[22]. At the time of parturition, lactogenesis phase II is initiated.

Lactogenesis phase II is characterized by the expression and upregulation of additional genes responsible for milk production in the alveolar cells and the onset of colostrum formation. Soon thereafter copious production and secretion of mature milk ensues after the closure of tight junctions between milk-producing epithelial cells in the full compliment of lobular alveolar complexes[22]. Lactogenesis phase II is established with high levels of prolactin and an immediate withdrawal of progesterone at parturition[22]. Phase II of lactogenesis is maintained by the constant removal of milk from the mammary gland lumen (suckling), high levels of prolactin (maintenance of milk production from alveolar cells), and a constant supply of oxytocin (contributing to the milk-ejection reflex by stimulating myoepithelial cell contraction). The regulation and feedback patterns of each lactation hormone are great and will not be discussed here in any detail; however, Table 1.1 provides a summary of the responsibilities of each hormone during lactogenesis.

Milk synthesis and secretion places tremendous energy demands on the mother. All mammals have developed their own “lactation strategy” to meet these high-energy

demands, regardless of each animals' lifestyle during the lactation period[25]. Animals who remain sedentary and do not eat for the duration of the lactation period most certainly employ a much different strategy than those animals that maintain eating and/or hunting practices over this period, as these non-fasting animals can rely on both endogenous energy stores and exogenous energy sources to provide the fuel required for milk synthesis[25].

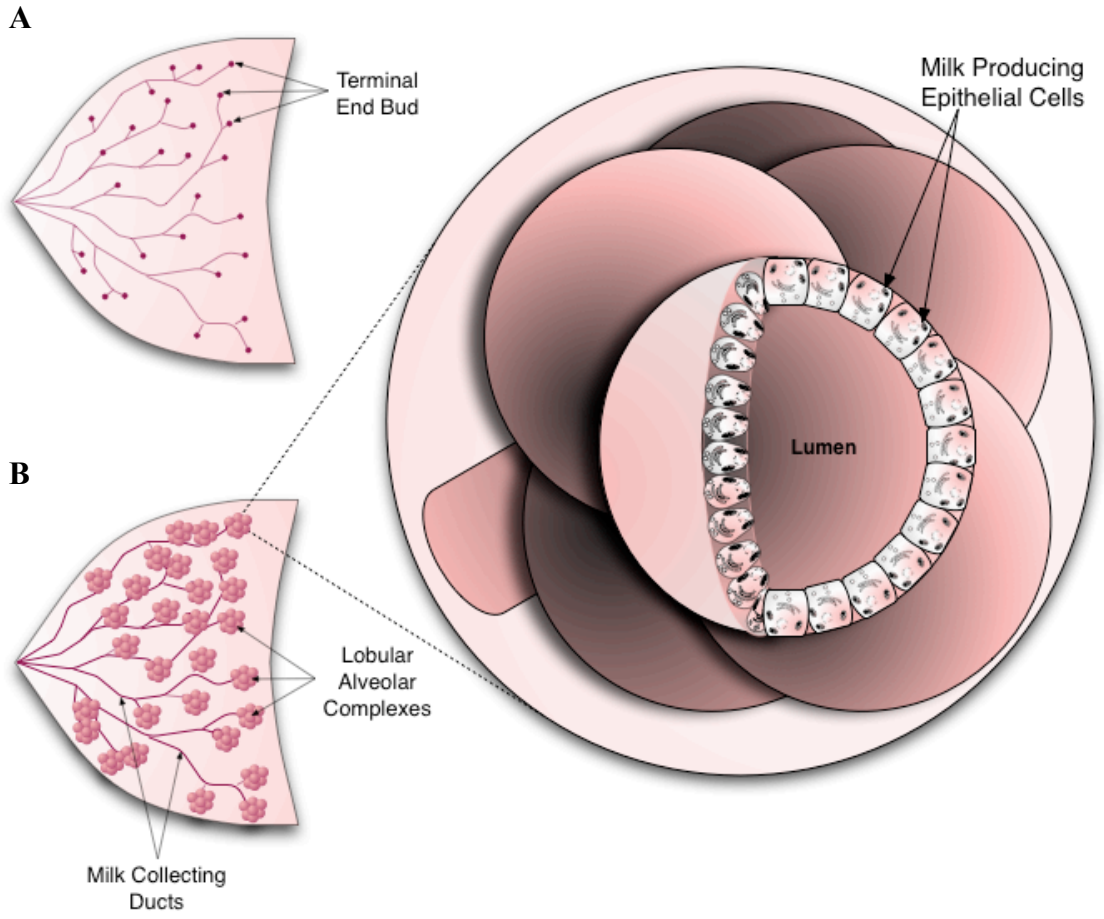


Figure 1.1 – Simple representation of the mammary gland structure in the virgin state (A) and the remodeling that occurs during lactogenesis (B) with emphasis given to the mammary alveoli (insert from B). Each lobular alveolar complex is composed of clusters, primarily of milk-producing mammary epithelial cells, which secrete milk into a defined lumen to be transferred into milk collecting ducts and excreted from the breast during breastfeeding. The figure is presented to emphasize the changes in mammary alveoli and does not capture the adipose tissue, blood supply, or myoepithelial cell composition of the mammary gland.

Table 1.1 – Roles and responsibilities of hormones released during lactogenesis phase I and II[22].

Hormone	Responsibility
Reproductive Hormones	
Estrogen	Ductal morphogenesis & possible induction of prolactin
Progesterone	Alveolar proliferation in lactogenesis I and withdrawal stimulates lactogenesis II
Placental Lactogen	Support of corpus luteum during lactogenesis I
Prolactin	Alveolar proliferation & maintenance of lactation during lactogenesis II
Oxytocin	Milk-ejection reflex during lactogenesis II
Metabolic Hormones	
Growth Hormone	Ductal morphogenesis through IGF-1
Corticosteroids	Increase during lactogenesis I and II, possible role for nutrient flux
Thyroid Hormones	Increase milk protein synthesis & enhance receptiveness to growth hormone and prolactin
Insulin	Unknown. Possible substitute for IGF-1 (in culture)
Mammary Hormones[a]	
Growth Hormone	Ductal morphogenesis through IGF-1
Prolactin	Maintenance of lactation during lactogenesis II
Leptin	Fat mobilization during lactogenesis II
PTHrP	Mobilizing bone calcium during lactogenesis II

[a] During lactogenesis, the mammary gland becomes an endocrine organ, synthesizing it's own compliment of hormones for local paracrine or autocrine roles.

Adipose tissue is the major energy-dense tissue with fatty acids providing more than 2-times the energy per gram than any other energy source (carbohydrates and proteins). Fatty acids then play a leading role in providing the energy requirements to drive milk production and sustain lactation[25], and therefore females have adapted mechanisms to store copious amounts of fat during gestation that allow a distinct evolutionary advantage[25]. Whereas some animals must rely on an environment with an

abundance of resources, we are able to reproduce in environments even where resources run low. Even though humans reproduce and thrive in environments where resources are plenty, the copious amounts of adipose tissue accumulated during pregnancy greatly contribute to neonatal fuel sources, a way of optimizing maternal resources.

The maternal system must use both stored and exogenous resources to drive many physiological processes, which provide the neonate with adequate nutrients in a manner as to not completely disrupt maternal stores[25, 26]. Therefore, the mother must adapt other mechanisms that will provide adequate amounts of critical nutrients to overcome the high-energy demands of breastfeeding. Significant changes occur in the gastrointestinal tract of the mother throughout lactation. Gastric emptying rate, and gastrointestinal transit are significantly increased during the first 2 weeks of lactation[27] to accommodate the large nutrient demands of the nursing neonate. In addition, the mucosal surface area of the maternal small intestine is significantly enhanced in the proximal duodenum and decreases distally[28]. The increased surface area in the duodenum indicates a much greater capacity to absorb dietary nutrients. In fact, the majority of weight retention in mice after parturition was attributed to the increase in splanchnic organ mass and these mice showed a significantly increased ability to transport D-glucose, D-fructose, and L-proline[29]. In addition to macronutrient transporters, intestinal transporters responsible for the uptake of micronutrients such as calcium[30] and zinc[31] are also significantly upregulated during lactation. Furthermore, the intestinal multidrug resistance like protein member 2 (Mrp2; a member of the ATP-binding cassette transporter family (ABC)), responsible for the efflux of various xenobiotics into the intestinal lumen, is significantly increased throughout the lactation period indicating an adaptive mechanism to decrease the transport of dangerous chemical entities into maternal blood to be delivered to the neonate[32]. Many other pharmacokinetic parameters are also increased during lactation in addition to the changes in gastrointestinal absorption. Increases in plasma protein levels, cardiac output, total body water, and total blood volume will ultimately affect the appearance of both wanted, and unwanted compounds at the mammary epithelia[33] where all nutrient components of milk are secreted. The mammary gland has adapted numerous transport mechanisms that accommodate milk composition, which include major macronutrients

such as the various amino acids and glucose, and also the micronutrients such as the numerous milk ions, where the transport systems regulating the appearance of these nutrients has been extensively reviewed[34]. Recently it was also established that these transport systems do change throughout lactation as they contribute to a specialized breast milk formulation[3]. The contribution of these transport systems to milk composition will be discussed shortly. However, it appears from a recent report[3] that the regulation of transporter expression of the SLC and ABC transporters is not only complex, but highly structured such that all the resources of the epithelial cells are dedicated to providing nutrient-rich secretions. Genes that do not contribute to nutrient secretions (i.e. MDR & MRP) are not supported during the high-energy demands of lactation[3].

The maternal system has adapted many mechanisms during lactation to not only provide the neonate with vast amounts of critical macro- and micronutrients for growth and development without compromising maternal homeostasis, but it has also evolved mechanisms to limit infant exposures to potentially harmful xenobiotics.

1.2.2. Messages in Milk – Breast is Best

Lactation is a continuation of the "crosstalk" between mother and neonate seen during gestation. However, during lactation the mother may now be the dominant driving force governing infant nutrition through the composition of the breast milk, rather than the bi-directional transfer of nutrients across the placenta during gestation. Maternal milk is an extremely complex biological fluid that is composed of thousands of nutrient and non-nutrient components that compartmentalize in one of the phases of the milk; aqueous phase, colloidal dispersions of casein molecules, emulsions of fat globules, fat-globule membranes, and live epithelial cells[35], each of which play a key role in infant development. Furthermore, the nutrients present in human milk (i.e. iron[36], zinc[37], proteins/growth factors[38]& fats[39]) are significantly better absorbed in the neonate making it far superior to infant formula due to the unique composition and easy digestibility[40].

1.2.2.1. Critical Milk Constituents

1.2.2.1.1. Macronutrients

Macronutrients are nutritional components of the diet that can independently and/or collectively provide the metabolic energy necessary to maintain all metabolic functions in a living organism. These components include, but are not exclusive to the proteins, carbohydrates, and fats as the major nutrient substrates in our diets. Macronutrient roles in the neonate have been largely elucidated and any disturbance in the balance of these nutrients in the breast milk has very detrimental outcomes in the nursing infant[15-17, 19, 41, 42]. For instance, rat pups exposed to low or no-protein breast milk demonstrate a significant and permanent decrease in feeding pattern through altered circulating insulin and leptin levels[42], and a significant alteration in pancreatic β-cell function causing permanent, irreversible effects on glucose homeostasis[15, 41]. In addition, mothers exposed to a high carbohydrate diet during the lactation period passed on the high carbohydrate phenotype (chronic hyperinsulinemia and adult-onset diabetes) to the progeny indicating a permanent programming of β-cell function[19]. A number of studies have evaluated the nutritional excess or deficiency of macronutrients. The findings of these studies have been extensively reviewed[43-46], and is not the focus of my thesis work, rather my work will focus on the micronutrients.

1.2.2.1.2. Micronutrients

Unlike macronutrients, the micronutrients are needed in only very minor quantities in the diet to maintain health but do not directly contribute to overall metabolic fuel. Rather micronutrients contribute to a number of biochemical pathways. These components include vitamins, minerals, nucleosides and nucleotides, smaller peptides, anions, and cations[47]. Deficits or excesses of micronutrients are equally as detrimental as those of the macronutrients and only recently are we uncovering the effects of some of these micronutrient deficiencies or excesses during lactation due to maternal health status and the effect on the developing offspring[48]. The greater numbers of studies evaluating micronutrients are focused primarily on essential metal[49-54] and vitamin[48] excess or deficiency in early life development, however deficiencies in other micronutrients are well documented. Although the immediate effects of certain deficiencies are limited and are only visible in later life, other critical micronutrient

deficiencies (i.e. metal[55] or vitamin D[56]) are immediately visible and cause irreversible damage. Findings from such studies is driving research into exogenous maternal supplementation of certain vitamins and trace elements[49] to compensate for possible maternal deficiencies in these trace compounds. However, without a proper knowledge of the exact requirements of these components, supplementation guidelines may be misleading[47]. Unfortunately, micronutrient deficiencies in the mother are not always correlated with similar micronutrient deficiencies in the breast milk and therefore, may be difficult to detect. For instance, milk iron concentrations remain unaltered until the deficiency/excess are extreme[52], however even minor iron deficiencies cause an altered expression of the major milk proteins, the whey-acidic protein (WAP) and β-casein[57]. Therefore, monitoring milk nutrient levels may not always reflect maternal nutrient deficiencies. However, these nutrient deficiencies can potentially alter maternal milk concentrations, even when deficiencies are minor[57]. Furthermore, we lack a basic understanding of the immediate roles of other critical micronutrients in the mammary gland, if any, and how altered breast milk levels may influence the proper ontogeny of the neonatal system.

1.2.3. Transport of Milk Constituents

1.2.3.1. Mammary Gland Secretion Routes

The macro- and micronutrients exploit a variety of transport routes across the mammary epithelia into the breast milk. The excretion of these critical milk constituents is dependent on an established cellular architecture within the epithelium of the mammary gland; specifically the formation of new cytoskeleton components, interactions with neighboring cells (formation of tight junctions), and interactions between the epithelial cell and the extracellular matrix proteins[58, 59]. Once this architecture is established, the mammary epithelium becomes an extremely efficient medium to transport constituents into and out of breast milk. Each one of the critical nutrient and non-nutrient components of breast milk reaches the mammary gland lumen by its own unique entry process: I Milk fat globule secretion, II paracellular pathway, III protein-mediated transport pathway, IV exocytotic pathway and V the transcytotic pathway (Figure 1.2)[60].

The majority of literature to date discusses the changes that occur during lactation to only few of these major pathways such as the paracellular pathway, transcytosis of large molecules, milk-fat secretion, and protein-mediated transport of macronutrients[60-67]. However, with the exception of essential metal transporters[52, 53, 68-71], the changes in the protein-mediated pathways with respect to micronutrient elements have been largely overlooked. Therefore, the remainder of my thesis will focus attention on the protein-mediated transport pathways (pathway III; Figure 1.2) of micronutrients and put into perspective how the marked dynamics of this pathway during lactation make it a critical pathway in determining the highly specialized micronutrient composition of maternal milk.

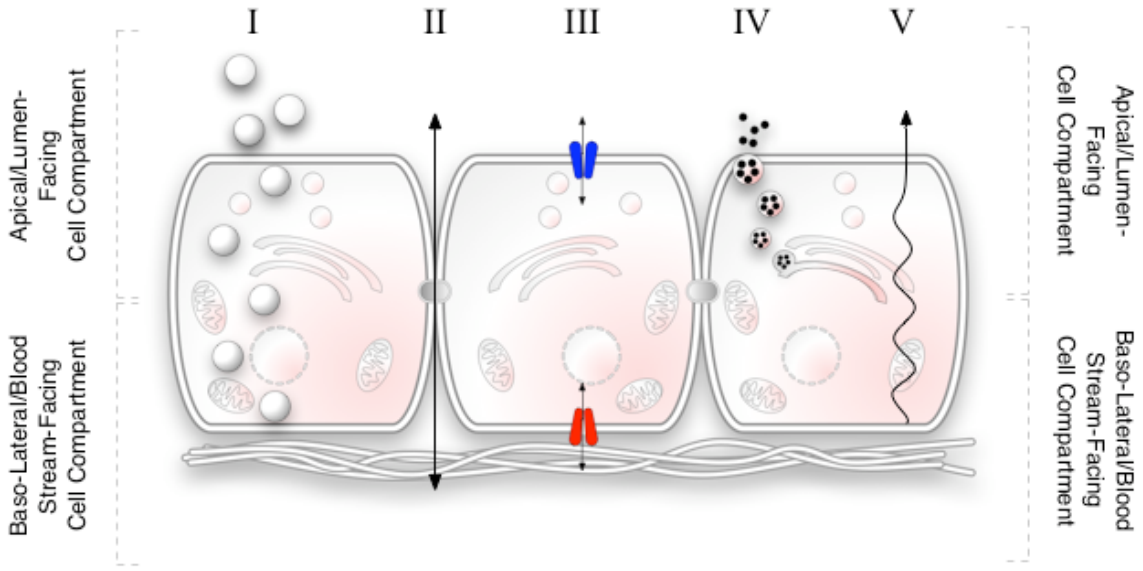


Figure 1.2 – The major secretion mechanisms of milk components from the polarized epithelium of the lactating mammary gland. I Milk fat globule secretion, II paracellular pathway*, III protein-mediated transport pathway, IV exocytotic pathway, and V transcytotic pathway. *Paracellular movement of solutes is limited to very early lactation during colostrum formation, however after the formation of tight junctions and the formation of mature milk, the paracellular pathway is non-existent for most solutes.

1.2.4. Transporter Expression in Lactating Mammary Epithelial Cells

Hormonal regulation causes significant changes in the mammary gland of pregnant mothers including cellular proliferation and differentiation into a polarized epithelium, the onset of milk production from mammary alveolar epithelial cells[22, 72] and

significant changes in the transporter mosaic that allows the transport of nutrients and endogenous compounds across the mammary epithelium[3]. Studies have shown the altered expression of many transporters associated with the mammary gland, specifically the altered expression of nutrient transporters that allow the passage of nutritive elements into maternal milk[3, 34, 52, 61, 67, 73-76]. A recent study[3] showed that human lactating mammary epithelial cells express a much different transporter mosaic than do non-lactating mammary epithelium. This altered expression mosaic includes changes in transporter mRNA expression levels of members in both the ATP-binding cassette transporter (ABC) family and the solute-carrier transporter (SLC) family.

1.2.4.1. ATP-Binding Cassette Transporters (ABC)

The ATP-binding cassette transporter superfamily consists of transporters that are characterized as primary active transporters, which use the coupling of ATP to drive the movement of substrate against or independent of a concentration gradient (Figure 1.3). These transporters are primarily efflux transporters, and can be expressed at the apical or basolateral plasma membrane compartments of a polarized epithelium and contribute to the removal of large lipophilic compounds from the cell and deposit them into a lumen or back into the blood stream[77].

Depending upon their localization in a polarized epithelium, then, these transporters can either accumulate compounds in a particular tissue or play a protective function by impeding the access of compounds to certain tissues, creating a privileged, protected compartment. For instance, P-glycoprotein (MDR) and the multidrug resistance-like proteins (MRP), both major players in xenobiotic disposition, are dynamically expressed at critical physiological barriers such as the gastrointestinal tract and blood-brain barrier, and contribute to multidrug resistance by governing the active efflux of xenobiotics[78-81]. Therefore, the expression of these proteins in the mammary gland will provide insight into the susceptibility of the mammary gland to xenobiotic attack, where the decrease in expression throughout lactation may be correlated with a decline in the ability of xenobiotics to penetrate the mammary gland lumen.

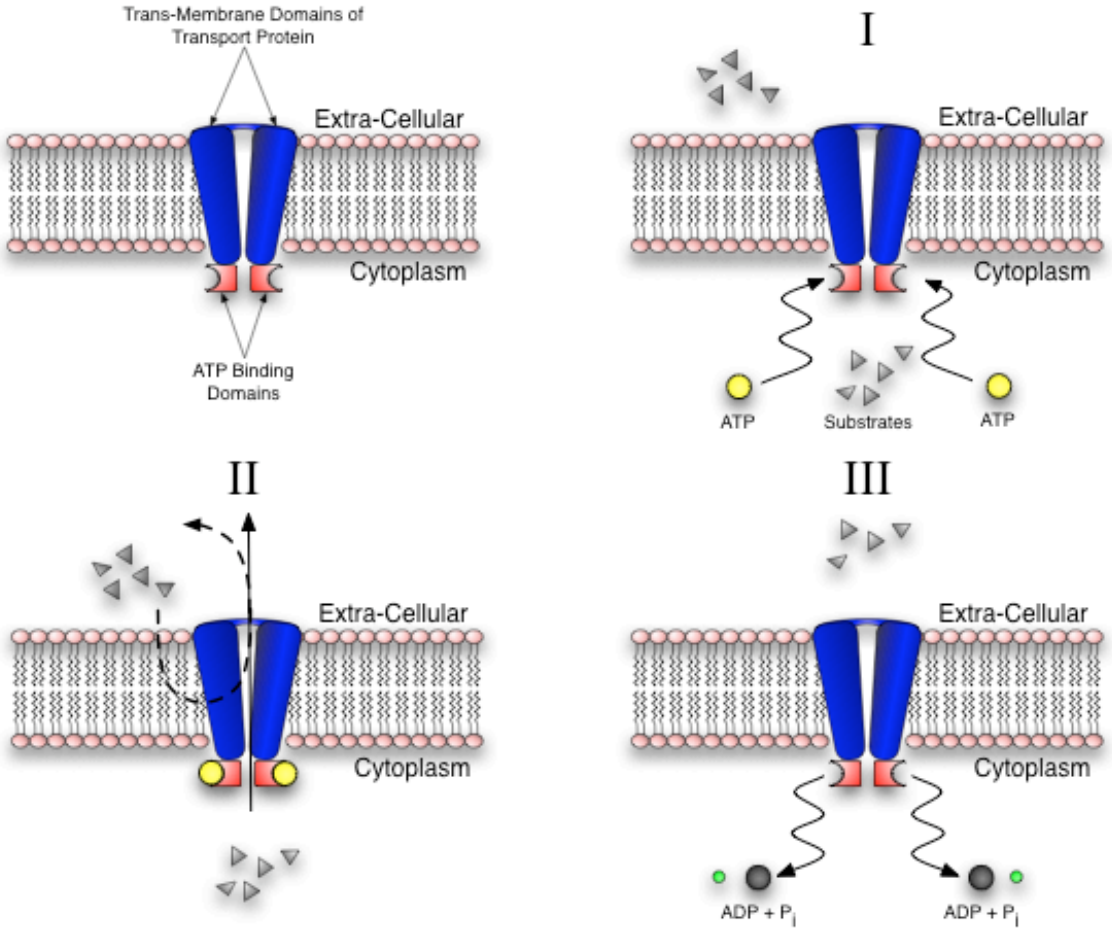


Figure 1.3 – A simplified representation of the mechanism of substrate removal from the cell by the ATP-binding cassette superfamily of proteins. I, the binding of ATP to the ATP-binding domains of the protein; II, substrate movement across the plasma membrane; III, ADP and inorganic phosphate are released from the ATP-binding domains and the cycle can be repeated. The active site for substrate binding is postulated to exist within the lipid bilayer (dashed arrow).

1.2.4.2. Solute-Carrier Transporters (SLC)

Classified as secondary or tertiary-active transporters, the transporters in the SLC transporter superfamily do not directly use ATP as a driving force for the movement of substrates, but rather take advantage of a previously established concentration gradient within the cell, or they actively create a concentration gradient for the transport of the desired substrate. SLC transporters can be facilitators (allowing for solute movement into and out of the cell based on an existing concentration gradient), exchangers/antiporters (removal of a compound drives the uptake of the wanted

substrate), or cotransporter/symporters (simultaneous transport of a compound that creates an energy-driving force and the wanted substrate; Figure 1.4)[82].

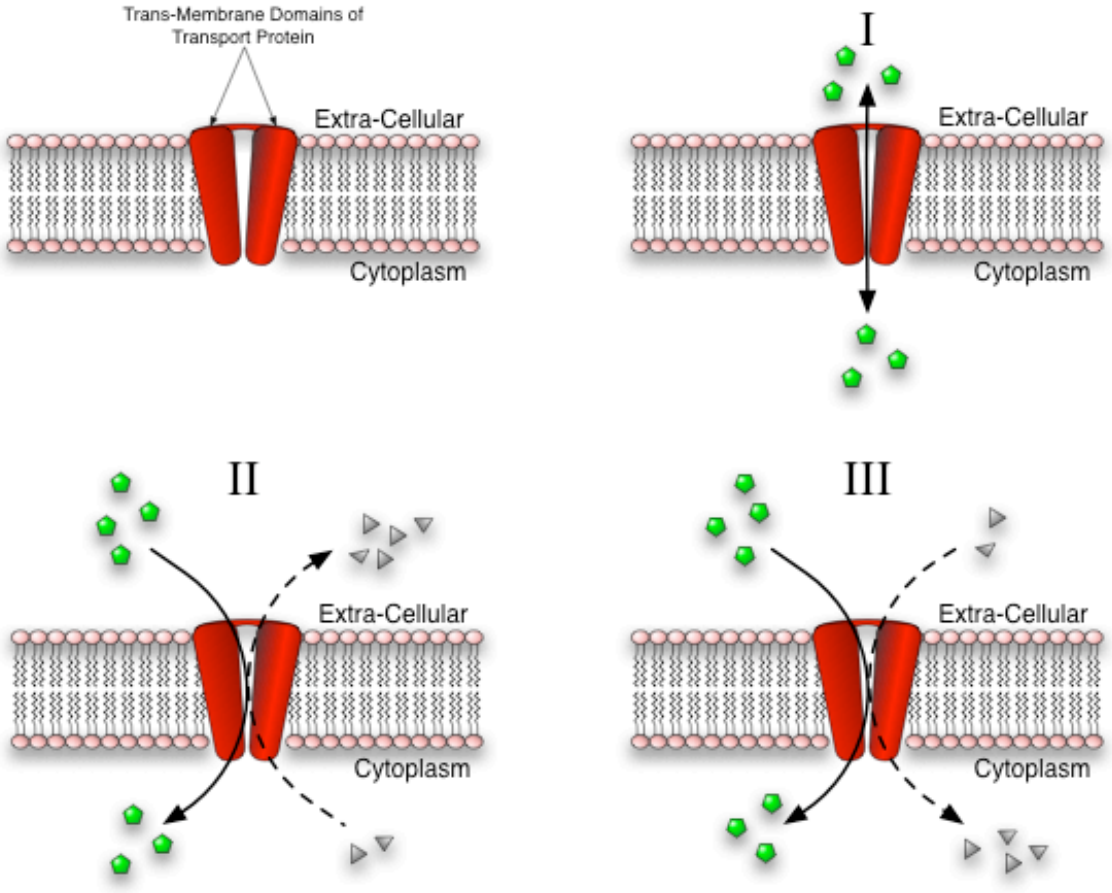


Figure 1.4 – A simplified representation of the mechanisms of substrate uptake by members of the solute carrier superfamily (SLC). I facilitated transporter, II exchangers/antiporters, III cotransporter/symporter. Arrows indicate the direction of movement of the desired substrate (solid arrows; green substrate) and secondary-active solute (dashed arrows; grey solute).

The SLC proteins are classified according to their endogenous role of solute transport, and consist of the following major families of transport proteins: Organic anion transporters; organic cation transporters; nucleoside/nucleobase transporters; oligopeptide transporters; and essential metal transporters[3, 83, 84]. These transporters are primarily uptake proteins and can be expressed at either the apical and basolateral compartments of a polarized epithelium. For instance, the concentrative nucleoside transporters are expressed at the luminal surface of absorptive epithelia (i.e.

gastrointestinal and renal barriers) and contribute to the uptake of critical nutrient components[85], whereas the equilibrative nucleoside transporters are expressed basolaterally in these epithelial barriers and contribute to the bi-directional transport of nucleosides[86]. Therefore, the expression of these transporters in the mammary gland may provide insight into their transport roles throughout lactation.

1.2.4.3. Transporter Role in Nutrient Transport into Lactating Mammary Epithelial Cells

The roles and cellular localization (apical or basolateral cell compartment) of both the ATP and SLC superfamilies in solute transport have been elucidated for many polarized epithelial barriers (i.e. kidney, liver, blood-brain barrier, intestine, lung, etc.) in many different species[87]. The cellular localization of the protein combined with the knowledge of the protein function gives insight into the role of this protein in the accumulation and/or removal of specific substrates from the cells at each barrier. However, despite the large amount of effort put into the evaluation of these transporters, there has been very limited work dedicated to the cellular localization of these transporters in the mammary gland. Knowledge of the movement of specific constituents from the blood stream into maternal milk (or visa versa) based on cellular localization would prove to be an invaluable resource.

Each nutrient transporter has a specific substrate profile and role that is reflected in its nomenclature (Table 1.2)[83, 84]; however, this information is inadequate in completely determining how these proteins contribute to the composition of maternal milk. The missing puzzle piece is, of course, the cellular localization of these transporters and their expression levels during different stages of lactation. As stated previously, localization and function taken together can provide insight into the role that a transporter will play in a given tissue or barrier. Figure 1.5 shows the cellular localization of transporters from both the ABC and SLC transporter superfamilies in a typical polarized barrier epithelium.

Table 1.2 – Nutrient substrate profiles and mechanism of action for transporters from the ATP-binding cassette (ABC) and the solute carrier (SLC) superfamilies. A complete list of known SLC and ABC transporters is also available[83, 84]. This list identifies those transporters assessed in this thesis research.

Transporter	Function/Coupling Ions[a]	Nutrient Substrate Profile
Mdr1a	Efflux/ATP	Unknown
Mdr1b	Efflux/ATP	Unknown
Mrp1	Efflux/ATP	Unknown
Oct1	F	Organic cations[88]
Octn1	F or E/H^+	Organic cations/carnitine[88]
Octn2	C/Na^+	Organic cations/carnitine[88]
Octn3	C/Na^+	Organic cations/carnitine[89]
Cnt1	C/Na^+	Pyrimidines & adenosine[85]
Cnt2	C/Na^+	Purines, uridine[85] & adenosine[90]
Cnt3	C/Na^+	Purines & pyrimidines[85]
Ent1	F	Purines & pyrimidines[86]
Ent2	F	Purines, pyrimidines, & nucleobases[86]
Ent3	Unknown	Purines, pyrimidines, & nucleobases[86]
Ncbt1	C/Na^+	L-Ascorbic acid & nucleobases[91]
Ncbt2	C/Na^+	L-Ascorbic acid & nucleobases[91]
Pept1	C/H^+	Di- & tri-peptieds[92]
Pept2	C/H^+	Di- & tri-peptides[92]
Mtx1	C/Na^+	Folate/methotrexate[93]
Dmt1	C/H^+	Non-heme (ferrous) iron[94]

[a] (F facilitated transporter, E exchanger, C cotransporter)

Although Figure 1.5 shows cellular localization of ABC and SLC transporters in a typical polarized barrier epithelium (i.e. intestine, kidney, liver, brain), it may not accurately reflect the localization of these transporters in the mammary epithelium. For instance, the multi-drug resistance like protein, Mrp1 has different cellular expression patterns depending on the polarized barrier tissue. Mrp1 is expressed apically at the

blood-brain barrier, however expressed at the basolateral membrane compartment in most other polarized barrier tissues (intestine, kidney, blood-testis, blood-CSF and blood-placental)[95]. Nevertheless, these transporters are present in the mammary gland[4], expressed at different levels between non-lactation and lactation states[3] indicating some sort of regulatory mechanism, and do transport critical micronutrients (Table 1.2) which contribute to the optimal composition of maternal milk.

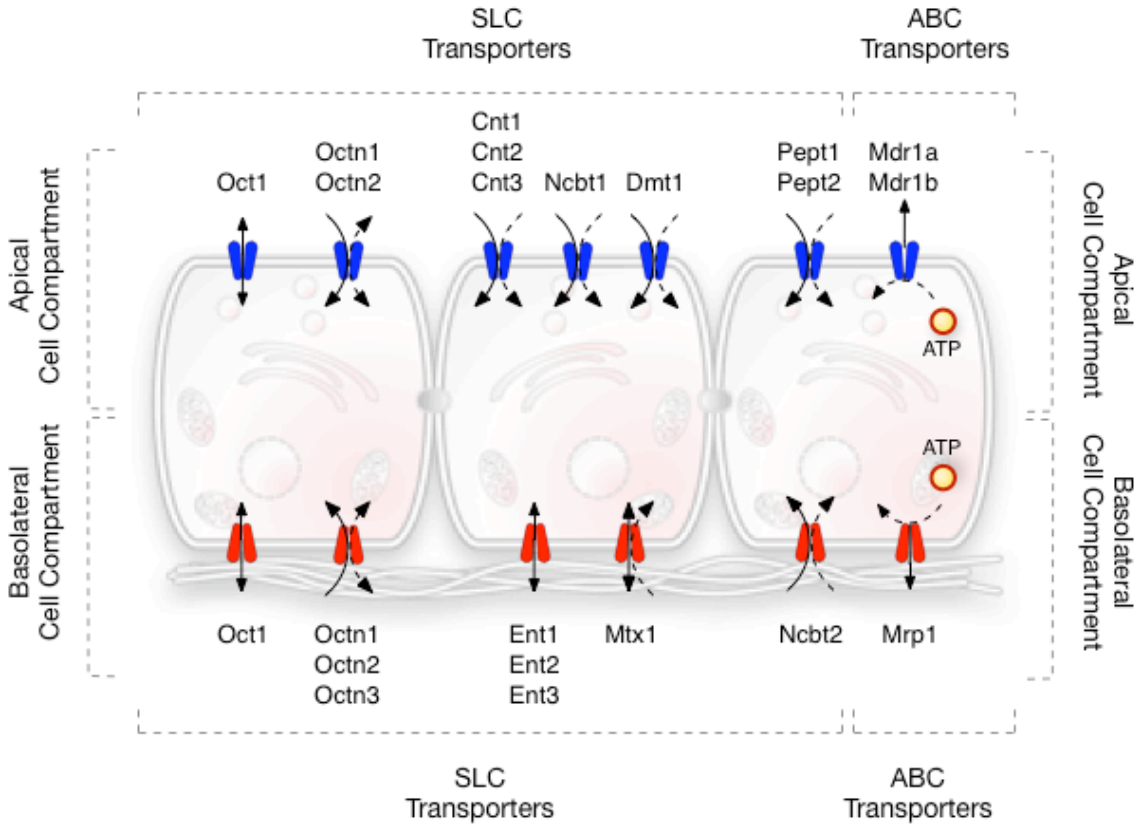


Figure 1.5 – Cellular localization of transporters from the ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies of transport proteins in a typical polarized tissue. Transporters shown at both apical and basolateral surface are expressed at either surface depending on the tissue epithelium and are not expressed in both compartments in the same tissue.

1.2.4.4. Xenobiotic Interference with Transporter Function

All transporters have a preferred substrate profile; a profile that is grounded on physicochemical characteristics. Nevertheless, many foreign compounds (i.e. xenobiotics or environmental toxins) possess physicochemical characteristics similar to

the preferred substrates of a transporter. Similar characteristics between a preferred substrate and the foreign compound may result in a competition for the transporter active site, and if the foreign compound out-competes the substrate, it can lead to an unfavorable dynamic.

The classic example is the interplay between intestinal p-glycoprotein (MDR) and its enzyme-counterpart, CYP3A4. Inhibition of intestinal MDR or CYP3A4 function has lead to altered pharmacokinetics/toxicokinetics by eliminating the intestinal first-pass effect[96]. For instance, administration of 6',7'-dihydroxybergamottin (DHB), one of the components of grapefruit juice (a potent intestinal CYP3A4 inhibitor), along with a drug, significantly increased systemic drug levels which can lead to serious hepatotoxicity[97]. However, this inhibitory effect is not exclusive to xenobiotic transporters and can be seen in nutrient-specific transporters as well. For instance, the substrate profile of the organic cation/carnitine transporter, OCTN2, is nearly exclusive to organic cations and carnitine. However, clinically relevant drugs such as the β-lactam antibiotics[98] and the oral hypoglycemic agents, sulfonylureas (used in the management of type 2 diabetes)[99], are also substrates for OCTN2. These xenobiotics have the capacity to occupy the active site of OCTN2 in the renal proximal tubule, preventing the reabsorption of filtered carnitine resulting in an increased urinary carnitine excretion and ultimately an acquired carnitine deficiency[98, 99]. Carnitine deficiency can lead to many metabolic conditions including progressive cardiomyopathy, encephalopathy, and skeletal myopathies, often in a very short period of time (3-6 weeks)[20]. Acquired carnitine deficiency due to xenobiotic interactions with OCTN2 is a particularly poignant example of a very serious adverse health outcome following a xenobiotic-nutrient transporter interaction.

Transporter inhibition by xenobiotics is not limited to drug-transporting proteins, and nor are these interactions limited to a single polarized epithelium. Therefore, it may be possible that a xenobiotic can interfere with transporter function at the mammary gland, resulting in an altered flux of nutrients across the mammary epithelia.

1.2.5. Benefits of Breastfeeding

> *"Human milk is species-specific, and all substitute feeding preparations differ markedly from it, making human milk uniquely superior for infant feeding"*[100]

The unique composition of human milk has evolved from our distant past to encompass a plethora of critical components in addition to nutrients, such as immune factors and hormones, that not only make it superior for infant nutrition, but also, overall infant health and wellness.[101, 102]

1.2.5.1. Health Benefits

1.2.5.1.1. Health Benefits to Nursing Infant

The unique species-specific components of human milk (hormones, immune factors, growth factors, and micro- and macro- nutrients) that arrive in the milk either in part or fully attributed to the function of mammary gland transport systems, provide the nursing infant with critical nutrient and protective factors, which leads to numerous health benefits associated with infant breastfeeding. There exists a myriad of published literature on the possible effects of exclusive breastfeeding on protection from acute and chronic illnesses, and its role in infant growth and development[7, 103-108]. Therefore the following section will only provide a small insight into the proposed claims that breastfeeding is protective and critical for optimal infant development.

1.2.5.1.1.1. Acute Disease

Acute infant diseases are often associated with the development of colic in newborns and can lead to serious health problems if left untreated[106, 107, 109, 110]. Recent longitudinal analyses of data shows that as little as a 2-month increase in exclusive breastfeeding (6 versus 4 months) significantly decreased the incidence of pneumonia and otitis media in newborns within the first year of life[109], and exclusive breastfeeding decreases the risk of developing acute gastrointestinal infections, urinary tract infections, and necrotizing entercolitis in newborns by an altered mucosal colonization pattern of gut microflora[110]. Exclusive breastfeeding has been shown to be protective against a host of other disorders including asthma and allergy[106, 107], however the literature

remains divergent.

1.2.5.1.1.2. Chronic Disease

The prevention of many chronic diseases such as cardiovascular disease, obesity, and diabetes has received a large amount of attention recently[111]. The onset of many of these diseases has been attributed, at least in part, by early life nutritional status[104, 105, 112-115].

The epidemic of adolescent and adult obesity in western society is constantly increasing and is one of the leading contributors to the development of cardiovascular disease, diabetes and insulin resistance, and early life mortality[111]. Recent reviews summarize the majority of literature and breastfeeding has shown to have a small yet positive effect on obesity in adult life as determined using body mass index (BMI) analysis[108, 116, 117]. Despite the attempt to control for all confounders, many of the studies indicate that the uncontrollable factors such as adult diet and genetic variability may offset the potential positive effects.

Significant differences in blood lipid profiles do exist between children who are exclusively breastfed and those who are prematurely weaned during the first year of life. Exclusively breastfed infants show a significantly increased accumulation of serum cholesterol, higher LDL cholesterol and apoprotein B concentrations, and higher levels of certain HDL cholesterols during the initial months of life[118]. However, meta-analyses following breastfed infants into later life have not been able to decisively predict the effect of breastfeeding on the risks of cardiovascular disease. Marked differences visible in early childhood have seemed to disappear in some investigations, as serum lipid profile differences do not last into later life [119]. However similar meta-analyses seem to maintain that breastfeeding and its duration actually improve blood lipid profiles, both in youth[120] and later adolescence and adulthood[121].

Similarly, the causality between exclusive breastfeeding and improved blood pressure in adolescence and adulthood are also misleading[121-125]. Longitudinal studies indicate a positive effect of breastfeeding on blood pressure; however, the magnitude of change is similar to that of a reduced salt diet[114] and may be a function of adult lifestyle and diet habits. On the other hand investigations question the experimental power and error in showing a causal relationship when controlling for all potential confounders[122].

Diabetes is a metabolic disorder characterized by persistent hyperglycemia commonly resulting from an autoimmune disorder attacking pancreatic β-cells and consequently an inadequate insulin secretion (type I), or a lifestyle-related disorder where there is an insensitivity to insulin at the target tissue, as well as inadequate β-cell function and insulin production (type II). Much of the literature states that there is a positive dose-response relationship between breastfeeding and the incidence/risk of type I diabetes mellitus (DM)[126-128]. For instance, a case-control study in Czech children[126] and Australian diabetes patients[128] showed a protective effect of breastfeeding duration on the development of type I DM that was unaffected by any confounding variables. However, other such studies indicate that the incidence of type I DM may not be attributed to duration of breastfeeding or the early introduction of cow's milk and/or solid foods, and may only be determined by a genetic predisposition[129]. The proposed protective effects of breastfeeding on the incidence of type I DM are extremely divergent and unclear.

In comparison to type I DM, there is far less literature associating breastfeeding duration on the risk of developing type II DM in later life. Breastfeeding does appear to be protective against developing type II DM[130, 131]; however, similar to type I DM, the confounding variables for developing type II DM are far too many to control and therefore the possible mechanisms governing the protective effects of breastfeeding on type II DM are also vague and unclear.

The potential protective effect of breastfeeding on numerous infections begs the question to what extent breastfeeding may protect against childhood cancers. Meta-analyses show that breastfeeding may be protective against certain types of childhood cancer such as acute childhood leukemia[132, 133] and non-Hodgkin's lymphoma[134]. Nevertheless, meta-analyses done in other countries have shown no such correlation[135] indicating that there may be location-based confounders.

The positive effects of breastfeeding duration on the prevention of chronic diseases such as cardiovascular disease, blood pressure, and diabetes may be attributed (at least in part) to the programming of lipid metabolism due to the higher fat content of breast milk compared to that of formula and hence the positive effects on later life obesity. However, much experimentation is needed to determine if there exists such a

causal relationship.

1.2.5.1.1.3. Growth & Development

Breast milk contains a plethora of essential components critical for proper growth and development, such as the long-chain polyunsaturated fatty acids (LC-PUFA). LC-PUFA are abundant in breast milk and play a key role in membrane structure and are used in extremely high amounts during brain and retina development[136]. Their role in brain and retina development has indicated them as primary players in proper overall infant growth and development.

Exclusively breastfed infants are significantly heavier and longer (length-for-age) than partially-breastfed or formula fed infants throughout the first 3 months life. However, this difference declined thereafter and by 12 months where weight and length were similar, independent of early life nutrition[137]. Also in premature infants, breastfeeding contributed to higher motor skill development at 3 and 12 months and higher cognitive scores at 12 months compared to formula fed infants at the same age[138]. Does breastfeeding only increase cognitive function in premature infants? In a retrospective study of Danish men and women, it was shown that breastfeeding did increase intelligence in adult life[139]; however, it appears that the results of many of these studies need to be critically evaluated before proper interpretation can occur. A recent review describes that in a sample of 40 publications between 1929 and 2001, only 2 publications met the proper standards of high-quality feeding data, and of those 2 studies the results were divergent[140]. Therefore, although results appear promising, they may also be misleading.

It has been well documented that exclusive breastfeeding has the possibility to protect against a myriad of childhood illnesses and diseases such as gastrointestinal tract infection/diarrhea, otitis media, upper respiratory tract infections, obesity, cardiovascular disease, diabetes, and certain cancers. However, such benefits may not necessarily represent our society on an individual level because the majority of studies are longitudinal in nature. These studies lack an exclusive causal relationship (due to numerous confounding factors) between individual breastfeeding practices/durations and health benefits. Nevertheless, the possible role that breastfeeding may play on immediate and/or future health and development status cannot be ignored.

1.2.5.1.2. Health Benefits to Mother

Although breastfeeding is considered to be optimal for overall childhood health and wellness, it offers many health benefits to mothers as well. Changes in post-partum, serum hormone levels, such as prolactin, oxytocin, estrogen, and progesterone have been shown, not only to regulate lactation status, and milk production[22], but also to regulate many other processes, which are beneficial to the mother. For instance, the increased serum concentrations of oxytocin are attributable to the increase in post-partum fat mobilization[22] and therefore the rapid return to pre-pregnancy weight. In addition, prolonged breastfeeding has also been suggested to protect against certain cancers such as pre-menopausal, but not post-menopausal breast cancer[141], with mechanisms unknown.

1.2.5.2. Social Benefits

In addition to the immediate and long-term health benefits of breastfeeding to both the mother and the nursing infant, both parties also benefit from the close, social interaction during breastfeeding. The long periods of maternal care allow the passage of maternal influences onto the nursing young. This "privileged" interaction between mother and nursing infant provides a feeling of safety, comfort, and well being for both mother and infant[2, 142]. For mothers suffering from post-partum depression, breastfeeding allows them to focus on a very positive experience, and the increase in maternal oxytocin levels during this time can help reduce the effects of depression[143]. Furthermore, the close skin-to-skin interaction (also known as "kangaroo care"[144]) between mother and infant has been shown to be analgesic. Infants exposed to a heel-lance, a routinely used hospital procedure to collect blood, showed a major decrease in pain response by upwards of 91% as indicated by outcome measure of crying and grimacing[145, 146]. This study also showed that both resting and stressed heart rates were reduced, confirming a possible analgesic role of breastfeeding for the nursing infant. In addition, the close skin-to-skin contact between mother and infant has been shown to be a good mediator of infant body temperature[147]. This skin-to-skin contact may subconsciously increase the awareness of the infant to mother and visa versa and may ultimately help to stimulate breastfeeding in couples experiencing breastfeeding difficulties[147].

1.2.5.3. Economic Benefits

The ability of women to breastfeed represents a significant economic benefit to both the private and public sectors of health care and economy. If breast milk is considered a food commodity and therefore a product that contributes to the total food supply (gross domestic product (GDP)), breastfeeding demonstrated a net economic benefit of $2.2 billion/year in Australia alone[148]. Such an economic benefit should prove to be extremely beneficial to the economy of any nation. However, even if breast milk is not considered as a food commodity, it still holds many benefits. The ever-rising cost of infant formula puts a real strain on those mothers who do not breastfeed by choice or by advice of a physician, specifically low-income and younger mothers who are unable to invest extra resources into specialized infant formulas.

The positive health benefits associated with breastfeeding such as the decreased risk and severity of childhood illnesses reflects a decreased stress to the personal and public health care system by lowering the incidence of visits to pediatric physicians and hospital stays. During the first year of life, there were 2033 additional office visits, 212 additional hospitals stays and 609 additional prescriptions written for each 1000 never breastfed infants when compared to infants who were exclusively breastfed for only 3 months[149]. The additional health care services for non-breastfed infants cost the health care system between $331 and $475 per never-breastfed infant during the first year of life in the United States[149].

1.2.5.4. Endorsement & Promotion of Breastfeeding

Recently, an increased effort to promote and endorse breastfeeding has occurred due to the health, social, and economic benefits of breastfeeding. Many governing agencies (U.S. Food and Drug Administration, Public Health Agency of Canada, Canadian Pharmacists Association, American Academy of Pediatrics, World Health Organization) publish yearly position statements on the promotion of breastfeeding practices and the use of human milk in infant feeding[8-12]. These statements cover all major areas including feeding frequency and duration of feeding practices, pacifier use, proper feeding positions, and contraindications to breastfeeding. Despite the small discrepancies in the messages of various supporting groups, they each independently and collectively support exclusive breastfeeding for at least 4-6 months, and the continuation

of breastfeeding with the addition of solid foods lasting up to 1-2 years of age.

National initiation and duration rates are on the rise. Breastfeeding has been constantly promoted since the mid 1960's, when it was determined that human milk was far superior to artificial formulations. Recent reports from Abbott Laboratories suggest that the initiation of breastfeeding in 2001 reached an all-time high in the United States with ~70% of all mothers were exclusively breastfeeding in the early post-partum period. However, by 6 months post-partum, only ~30% of mothers were still exclusively breastfeeding[150]. Similar trends exist in Canada with initiation rates in Ontario in 2001 accounting for ~80% - 90% of all mothers at the time of departure from hospital, which decreased by ~25% by 4 weeks after discharge with those mothers switching to formula[151]. The percentage of mothers who initiate breastfeeding in the early post-partum period meets the benchmark expectations from the governing agencies; however, the duration of breastfeeding falls far below these expectations.

1.2.6. Risks of Breastfeeding with Exogenous Drug Exposures

Few situations exist where breastfeeding is contraindicated for infant health. Concerns arise though in the presence of maternal exposure to drugs. The two critical risks associated with intentional (drugs) or unintentional (environmental toxins) maternal exposures are milk levels of drugs/xenobiotics and the possibility of these exposures affecting mammary gland functions, such as altered hormone regulation, milk production, and milk composition.

1.2.6.1. Safety Evaluation for Drug Use in Pregnancy & Lactation

Many healthcare practitioners still rely solely on reports from the American Academy of Pediatrics (AAP) and from a select group of researchers[8-14] to dictate the safety of breastfeeding practices with maternal medication use. These reports are based primarily on the appearance of parent drug in maternal milk and consequential neonatal exposure levels (exposure index). In some cases, safety recommendations are based on known, adverse effects on milk production. In 2002, the FDA sponsored a workshop mandated to provide information on the transfer of drugs into milk[152]. The primary findings from this meeting were that the following 3 items must be sufficiently evaluated in order to deem a drug safe for use in breastfeeding: maternal clearance (where an

increase in maternal clearance decreases neonatal exposure), neonatal clearance (where a decrease in neonatal clearance increases the exposure), and the possibility of active metabolites of parent compounds eliciting a response. None of these objectives attempt to characterize the ability of exogenous xenobiotics to influence the maternal milk composition, most notably because the function of transporters at the mammary gland has been, and continues to be, largely overlooked.

1.2.6.2. Drug Transfer into Maternal Milk

All compounds are present in human milk. The majority of drugs present in human milk arrive due to their physicochemical characteristics as they find passive equilibrium between maternal plasma and milk[153, 154]. Passive mechanisms, although being the most predominant mechanism of drug entry/exit from maternal milk, are not the exclusive mechanism to which chemical entities can transverse the polarized mammary epithelium. Milk-to-serum ratios have been observed at levels as high as 30 indicating that there are other mechanisms, such as active mechanisms present to transport drugs into maternal milk[155]. The precedence for the active transport of compounds into breast milk by transporters was recently established with the breast cancer resistance protein (BCRP), which is upregulated during lactation and actively accumulated toxicologically important substrates into the breastmilk[156]. Components of the active transport of xenobiotics into breast milk may also include constitutively expressed proteins from both the ABC and SLC families of transporters at the mammary epithelia, which include various nutrient transporters[3, 4].

Despite the specific nutrient-substrate profile of the nutrient transporters, some of these transporters also serve to transport many clinically relevant xenobiotics, some of which are not contraindicated during pregnancy and/or lactation[9-11]. Members of the organic cation/carnitine transporter family (OCT, OCTN), concentrative nucleoside transporter family (CNT), and equilibrative nucleoside transporter family (ENT) although specific for organic cations, organic cations/carnitine and nucleosides, respectively, they also serve to transport clinically relevant xenobiotics such as anticonvulsants, β-lactam antibiotics[157-160], acyclovir (ACV), gancyclovir (GCV) and zidovudine (AZT)[155, 161]. It may be possible that many other nutrient transporters have a substrate profile that encompasses other clinically relevant drugs.

1.2.6.3. Xenobiotic-Nutrient Transporter Interactions

It has been established that these "nutrient-specific" transporters also transport clinically relevant xenobiotics in addition to their nutrient substrates, so what happens when breastfeeding is initiated with an intentional or unintentional xenobiotic onboard? It is possible that the xenobiotic can out compete the nutrient substrate for the active site increasing the uptake of the xenobiotic, a phenomenon that is just recently being investigated with respect to one of the many mammary gland transporter families, the organic cation/carnitine transporter family[162]. However, this investigation and any others like it, only focus attention on the active transport of the xenobiotic into milk and consequent neonatal exposure to the unwanted compound. Little attention is being directed towards the possibility of this interaction altering the nutrient profile of maternal milk, specifically, the critical micronutrient composition of maternal milk where small alterations in these critical components may lead to long term metabolic regulation/programming.

1.2.7. Methods to Assess Transporter Expression, Function, & Interactions

It is becoming increasingly more important to fully understand the functions of transporters expressed at the lactating mammary gland to give insights into both mammary gland function/regulation and as a possible screening tool in predicting the ability of xenobiotics to influence the functions of these transporters. The use of *in vitro* cell culture model systems as an alternative to animal experimentation in basic science research has recently received much more attention as a possible screening tool for xenobiotic uptake and metabolism studies[163-166]. *In vitro* model systems are far more cost effective, have higher throughput, allow for greater replicates (stronger statistical power), and they add simplicity and accessibility to the study, both of which are difficult to achieve with the complexity of *in vivo* animal systems.

Furthermore, the rat mammary gland shows remarkable similarities with mammary glands of human population with regards to mammary gland function (milk-to-serum ratios), similar functional assessments (with respect to xenobiotic transport processes), and similarities in neonatal requirements (strict breast feeding until weaning)[155]. In addition, rat models offer an animal model system that is extremely cost

effective and accessible, and allows for sufficient sample size that is easily manipulated to an extent far beyond that of other tissue sources (i.e. human[3]). Therefore, the female rat will serve as a relevant model system for the evaluate nutrient transporters at the lactating mammary gland.

1.2.7.1. Isolated Plasma Membrane Vesicles

Membrane fractions isolated from whole tissue samples is a simple system for the evaluation of transport mechanisms. The major advantages of this method lay in its simplicity compared to whole tissue counterparts, the ability to eliminate metabolism as a confounder, and the ability to alter the fluid composition on either side of the membrane[167, 168]. However, these advantages are also major disadvantages when attempting to extrapolate results to *in vivo*, or even isolated, whole organ systems. Furthermore, membrane heterogeneity becomes a major disadvantage[167] and the composition of gradient media can further influence vesicle yeild[169]. Therefore, these systems are primarily used to obtain information regarding the affinity (K_m) and capacity (V_{max}) of various transporters[167, 168], much of which has been largely established with respect to secondary-active, nutrient transport[170].

1.2.7.2. Gene Expression Systems

Gene over-expression systems offer the advantage of inducing the expression of a specific transporter target[171]. The major advantage of gene over-expression, over that of isolated membrane vesicles, is the ability to precisely induce the target expression eliminating the variability of vesicle membrane composition. Furthermore, it allows for a more favorable cell condition as it maintains cellular architecture and can more accurately reflect the intracellular environment. However the disadvantages of this system are similar in that it is difficult to extrapolate results to *in vivo* systems[171].

1.2.7.3. Isolated Mammary Gland Perfusion

Isolated whole-organ perfusions have a main advantage in that they maintain the cellular architecture and *in vivo* environment of the organ. In the case of the mammary gland, which is grounded on the interactions between the milk-producing cells and their extra-cellular matrix environment[172-177], a whole organ perfusion would seem like a very reasonable system. However, aside from the direct advantage of this technique there are some important disadvantages that include the possibility of specialized surgical

techniques, the low throughput capability, and in order to maintain physiological conditions, the perfusion rate must match organ blood flow[178].

1.2.7.4. Cell Lines

In vitro cell culture systems have significant advantages over the complexity of an *in vivo* animal system; however, the major disadvantage to *in vitro* models is that the majority of current model systems utilize cultured cell lines as a model system, which may or may not be representative of the *in vivo* barrier. For instance, the highly used Caco-2 cell line used to study numerous carrier-mediated mechanisms, showed markedly different transporter expression levels than the gastrointestinal barriers *in vivo*[179] and therefore does not prove to be a relevant system when protein carrier-mediated mechanisms predominate. Therefore, any relevant in *vitro* model system must be validated using cells derived from the corresponding tissues to accurately reflect the *in vivo* barrier characteristics[164]. Currently, primary cell culture systems have been established for the evaluation of barrier functions at the blood-brain barrer[180], the blood-retinocyte barrier[181], and the blood-testis barrier[182], each of which have been shown to be more reliable for extrapolation of *in vitro* findings. Therefore a primary mammary epithelial cell culture would be far more reliable for the evaluation of the mammary gland barrier functions.

1.2.7.5. Total RNA Isolation From Whole Mammary Gland Tissue

The majority of reported literature suggests that the isolation of total RNA from a section of whole mammary gland tissue is an appropriate and accurate assessment tool to evaluate transporter expression levels[31, 52, 68-71, 73]. However, the mammary gland is composed of a heterogeneous population of cell types including adipose cells, myoepithelial cells, immune cells, milk-producing epithelial cells, and fibroblasts. A whole mammary gland preparation may not accurately reflect the true nature of the processes involved in the transport of solutes through the mammary gland epithelia into the breast milk, and consequently, a more relevant cell source such as a pure population of mammary epithelial cells would allow for far better assessments of transporter roles in the regulation of breast milk composition.

1.2.7.6. Primary Mammary Epithelial Cell Isolation & Culture

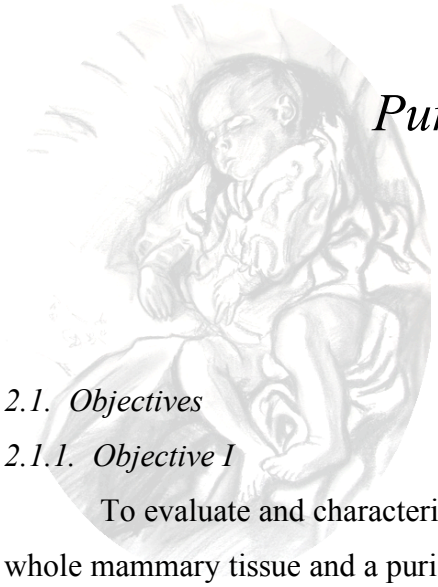
Isolated mammary epithelial cells from virgin or lactating animals, using various techniques (flow-cytometry[183], tissue digestion, or magnetic purification[184]) have been shown to develop into fully functioning alveolar-like structures after time in culture, if cultured appropriately on an extra-cellular matrix[185-189]. Cultures of this nature prove to be extremely beneficial as single cells grow into these fully functioning alveoli that are composed of a near pure population of milk-producing cells. Such characteristics facilitate the investigation into mechanisms occurring in the alveolar cells. However, disadvantages of this method exist beginning with the isolation method. Flow-cytometry isolation requires expensive equipment that may not be accessible to all individuals[190]; magnetic separation techniques (i.e. Dynabeads®[191]) are expensive and require specific antibody-bound magnetic beads to ensure a strict population of cells; and tissue digestion techniques involving a single-cell suspension require the use of enzymes that can be extremely harmful to cells[192]. In addition to the mechanical disadvantages, single cells must spend days in culture (10+) to develop into fully functional *in vitro* mammary gland[193].

1.2.7.7. Primary Mammary Epithelial Organoid Isolation & Cell Culture

Mammary epithelial organoids (or small organs) are clumps of alveolar cells and are quickly isolated from lactating mammary glands using enzymatic digestion[185, 194]. Isolated MEO are composed primarily of mammary epithelial cells[195], which are the functional cell type when assessing substrate movement across the mammary gland barrier. Therefore, appropriately cultured organoids share the same advantages as a primary culture involving single alveolar cells. However, organoids are isolated while functional and therefore are useful almost immediately after isolation. Therefore, organoids isolated from female rats will allow for better evaluations of transporter expression in lactating mammary epithelial cells. In addition, if MEO are appropriately cultured on an extra-cellular matrix in serum-free conditions, they will serve as a relevant *in vitro* system to evaluate nutrient transporter function and possible transporter-xenobiotic interactions.

1.2.8. Perspectives

In the parallel relationship that exists to assess and maintain neonatal health and safety there exists a crucial dyad: limiting drug exposures and assuring proper nutrition. Evaluation of drug levels in the breast milk (a measure of infant exposure), albeit a reasonable predictor of neonatal exposure to drugs, does not provide any assessment of the possible lack-of-exposure to critical micro- and macronutrients with such putative drug exposures due to interactions with mammary epithelial cell function. Therefore, the total assessment of neonatal safety with drug exposures *MUST* include the possibility of drug exposures altering the nutrient composition of maternal milk. It is well understood that active transport of drugs across membranes results in milk-to-serum ratios that can reach as high as 30 thus increasing the dose received by the nursing infant. Furthermore, these drug moieties are often substrates for nutrient transporters, and may competitively or non-competitively inhibit the transport of nutrients into maternal milk. The critical question is, then, under what circumstances will maternal exposure to drugs or environmental toxins pose a significant nutritional risk to the nursing neonate by interfering with proper mammary epithelial cell function? This thesis work provides an evaluation of the temporal expression patterns of various micronutrient transporters throughout lactation in the rat mammary gland. Furthermore, this thesis work will provide an assessment of when the mammary gland may be susceptible to a xenobiotic attack by exogenous compounds and, in addition, lay the basic groundwork for an *in vitro* model system that will, at its full capacity, provide an accurate assessment tool to evaluate xenobiotic-nutrient transporter interactions. This is only a small insight into a lifelong research endeavor; however, ensuring optimal breast milk composition is one additional measure in safeguarding overall infant health and wellbeing while breastfeeding.



- II -

Purpose of Project

2.1. Objectives

2.1.1. Objective I

To evaluate and characterize nutrient-transporter mRNA expression kinetics in whole mammary tissue and a purified population of mammary epithelial organoids (MEO) isolated from female Sprague-Dawley rats at various stages of pregnancy, lactation, and involution using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR). This objective will characterize the differences in expression patterns and levels between the literature "gold standard" (whole mammary gland tissue) and a plausible, more appropriate tissue source rich in milk-producing epithelial cells (MEO).

2.1.2. Objective II

To determine if appropriately cultured mammary epithelial organoids (MEO) maintain *in vivo* transporter expression to lay down critical groundwork for the development of an *in vitro* screening tool assessing xenobiotic-nutrient-transporter interactions.

2.2. Hypotheses

2.2.1. Objective I

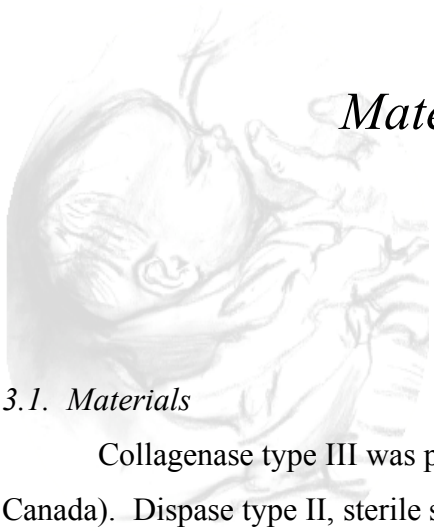
Nutrient transporter mRNA expression levels change throughout pregnancy and lactation in order to accommodate the nutrient composition of breast milk that serves the immediate needs of the developing nursing neonate. Furthermore, transporter expression levels are different in a tissue sample rich in secretory cells (MEO) than in whole mammary gland tissue.

2.2.1. Objective II

Isolated mammary epithelial organoids (MEO) cultured in an environment rich in extra-cellular matrix proteins (Matrigel™) maintain transporter expression at the mRNA level to serve as an appropriate system to evaluate xenobiotic-transporter interactions at the mammary gland.

- III -

Materials & Methods



3.1. Materials

Collagenase type III was purchased from Cedarlane Labs (Hornby, Ontario, Canada). Dispase type II, sterile saline (0.9%), fetal bovine serum (FBS), sterile 15 mL and 50 mL polypropylene centrifugation tubes, Falcon tissue culture flasks (T-150 & T-75), bottle-top filters (0.2 μm & 0.45 μm), and sterile polystyrene tissue culture dishes (100mm) were purchased from VWR (Mississauga, Ontario, Canada). Antibiotic solutions (antibiotic/antimycotic solution & gentamicin), DMEM-F12 cell culture medium, and phosphate buffered saline (PBS) were purchased from Invitrogen (Burlington, Ontario, Canada). Autoclavable Nitex filters (530 μm & 55 μm) were purchased from Sefar International (Ville St. Laurent, Quebec, Canada). QRT-PCR Smartcycler® 25 μL reaction tubes were purchased from Fisher Scientific (Nepean, Ontario, Canada), and 1-step Quantitect SYBR green RT-PCR kits and RNA isolation kits (RNeasy Midi and Mini kits) were purchased from Qiagen Inc. (Mississauga, Ontario, Canada). Tissue culture reagents and hormones (fatty acid-free bovine serum albumin (BSA), bovine insulin, human apo-transferrin, ovine prolactin, progesterone, hydrocortisone, ascorbic acid) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and mouse EGF was purchased from Chemicon International (Temecula, CA). Matrigel™, Matrigel™ thick-coated cell culture dishes (35 mm), and MatriSperse™

were purchased from BD Biosciences (Mississauga, Ontario, Canada). All other solvents and reagents were of the highest analytical grade available.

3.2. Animals

Female Sprague-Dawley Crl:CD BR rats (16-week virgin, or first time pregnant/lactating; $n = 4$ per group) were purchased from Charles River Canada (St. Constant, Quebec, Canada) and allowed at least a 3-day acclimatization period where they were housed in groups of 4 (pregnancy) or housed individually (lactation & involution). Animals were maintained in a temperature and humidity controlled facility (22°C ± 2°C) on a 12-hour light:dark cycle (0700h – 1900h), fed standard rat chow *ad libitum* and had free access to water throughout the study. On their respective day of pregnancy (gestational day 15 & 20), lactation (day 1, 3, 7, 10, 14, 18) or involution (24h after removal of pups), animals were deeply anesthetized using an overdose of isoflurane (as determined by lack of reaction to a hard toe pinch) and sacrificed by exsanguination and thoracic cavity opening. Abdominal mammary glands (bilateral abdominal, cranial inguinal, and caudal inguinal) were isolated and rapidly excised. A small section was removed from the mammary tissue and stored in RNA*later*™ (Ambion Inc., TX) until further processing while the remaining tissue was stored briefly in 0.9% saline containing 1000 U/mL penicillin G, and subjected to tissue digestion to prepare a cell suspension rich in mammary epithelial organoids (MEO). MEO's were either stored in RNA*later*™ as per manufacturers instructions on storage of tissue culture cells until subsequent RNA isolation or appropriately cultured on Matrigel™ coated cell culture plates. All tissues stored in RNA*later*™, were stored at -20°C until RNA extraction. All procedures for the care and use of the animals were in accordance with CCAC regulations and approved by the University Committee on Animal Care and Supply (University of Saskatchewan, Saskatoon, SK, Canada).

3.3. Mammary Epithelial Organoid (MEO) Isolation

MEO from excised mammary glands of pregnant (gestational day 15 & 20), lactating (days 1, 3, 7, 10, 14, 18), and postlactational (24h after removal of pups) female rats were prepared as per the method of Darcey *et al.*[185] with minor modifications

(Figure 3.1). Isolated MEO using this method have been optimized and shown to produce a tissue source enriched with milk producing, mammary epithelial cells[185, 194, 195].

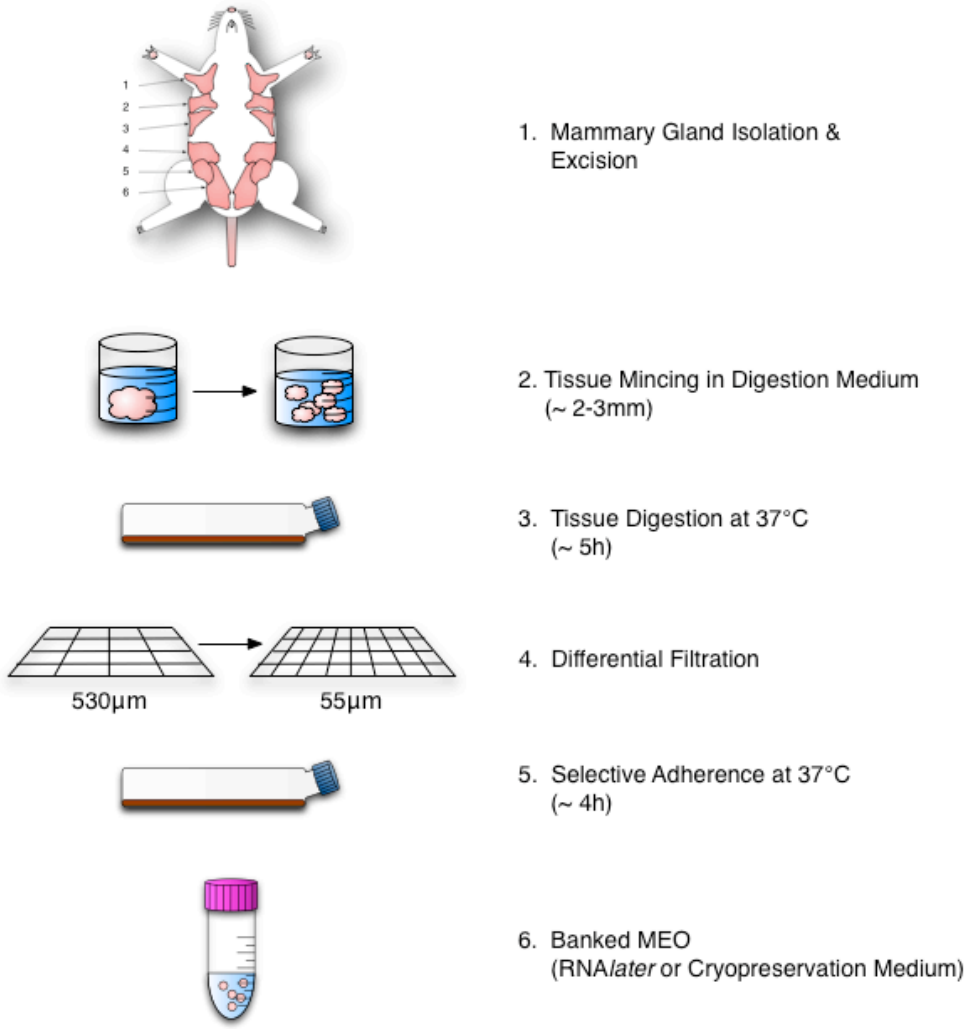


FIGURE 3.1 – Schematic representation of mammary epithelial organoid (MEO) isolation from excised whole mammary glands from a female Spraque-Dawley rat. MEO were isolated as per the method of Darcy *et al.*[185] with minor modifications. Refer to text for details.

Excised mammary glands were removed and stored in 0.9% saline containing 1000 U/mL penicillin G (Table 3.1), until all tissue fragments were collected. The collected mammary gland tissue was mechanically minced using sterile surgical scissors into uniform pieces (~1-3 mm^3) in a small volume of digestion media (~15 mL; Table

3.1) in a sterile 30 mL beaker. The fragments were incubated in digestion solution (10 mL digestion media/g mammary tissue wet weight) with minimal agitation at 37°C in a humidified CO_2 incubator for ~ 5 h (or until the majority of tissue had settled to the bottom of the flask as sand-like particles; MEO). The digestion solution and resulting MEO were split into sterile 50 mL polypropylene centrifuge tubes and pelleted by a 10 min 500 × *g* centrifugation at 4°C. The resulting pellet was washed once with 10 mL DMEM-F12 (Table 3.1), resuspended in 40 mL DMEM-F12, and filtered through a 530-μm Nitex filter to allow for the passage of MEO and single cells, and the removal of blood vessels and large tissue fragments.

The filtrate was filtered once more through a 55 μM Nitex filters to allow passage of single cells and the retention of MEO. MEO retained on the filter were rinsed off into a sterile tissue culture dish using a small volume of adherence media (Table 3.1) and transferred to a sterile T-75 tissue culture flask. The collected MEO were incubated at 37°C for ~ 3-4h with occasional gentle swirling to allow for remaining stromal contaminants and adiposities to adhere selectively to the flask. Nonadherent MEO recovered after incubation were transferred to a sterile 50 mL polypropylene centrifuge tube and pelleted by a 10 min 500 × *g* centrifugation at 4°C. The MEO pellet was washed once in 10 mL PBS, and resuspended in PBS:RNA*later*™ (1:9) as per manufacturers instructions and stored at -20°C to eliminate any possible RNA degradation until subsequent RNA extraction.

TABLE 3.1 – Detailed preparation instructions for solutions used in the isolation and culture of mammary epithelial organoids (MEO) from whole mammary gland tissue. Adapted from Darcy *et al.*[185]

Solution	Preparation	Storage Conditions
Primary Culture Medium	See Table 3.4	Prepared Fresh

Continued…

… Table 3.1 continued

Phenol Red-Free DMEM-F12 Medium	Supplement phenol red-free DMEM-F12 medium with 1.2 g/L sodium bicarbonate. Sterilize through 0.2 μm filter.	4°C
Saline Solution with antibiotic	Supplement 1 L of sterile 0.9% NaCl with 10^6 Units penicillin G	4°C
Digestion Solution	Phenol red-free DMEM-F12 medium supplemented with 0.2% (w/v) collagenase III, 0.2% (w/v) dispase II, 5% (v/v) FBS, and 50μg/mL gentamicin. Sterilize through 0.45μm filter.	Prepared Fresh
Adherence Medium	Phenol red-free DMEM-F12 medium supplemented with 5% (v/v) FBS, and 50 μg/mL gentamicin. Sterilize through 0.2 μm filter.	Prepared Fresh
Cryopreservation Medium	Phenol red-free DMEM-F12 medium supplemented with 50% (v/v) FBS, 10% (v/v) dimethyl sulfoxide (DMSO), and 50 μg/mL gentamicin. Sterilize through 0.2 μm filter before adding DMSO.	-20°C
Citric Acid Solution	0.1M citric acid in ddH_2O. Sterilize through 0.45 μm filter.	4°C
Nuclei Preparation Buffer	20 mM Tris (pH 7.4 at 4°C) with 3 mM $CaCl_2$, 2 mM MgCl, and 0.3% (v/v) Nonidet-P40. Sterilize through 0.2 μm filter before adding detergent.	4°C
Trypan Blue Solution	0.25% (w/v) trypan blue in ddH_2O. Sterilize through 0.2 μm filter.	4°C

3.4. RNA Isolation

Total RNA was isolated using RNeasy Midi isolation kits as per the manufacturer's directions. Tissue samples (~ 250 mg or 1 mL pooled MEO suspension) were homogenized in 4 mL of lysis buffer using a Polytron tissue homogenizer. The homogenized suspension was centrifuged for 10 minutes at 4500 × *g* to remove cellular debris and large tissue fragments. The resulting supernatant was removed, transferred to a new sterile polypropylene centrifuge tube, and 1 volume of ethanol (70% EtOH or 100% EtOH for whole tissue and MEO, respectively) was added to precipitate total nucleic acids. The sample was applied to the Midi column and cellular contaminants (including genomic DNA) were washed away using a series of buffers. The purified RNA sample was eluted from the column using a series of elutions using RNase-free water (250 μL). Total RNA was quantified by measuring the absorbance of a diluted sample (1:100 or 1:40 for whole tissue and MEO, respectively) of RNA:RNase-free water at 260 nm with a UV/VIS spectrophotometer (8453E, Agilent Technologies, Palo Alto, CA) according to the following equation:

$$[RNA] = 40_{\mu g/mL} \cdot A_{260} \cdot Dilution\ Factor \qquad [3.1]$$

RNA purity was assessed by measuring the absorbance ratio of a diluted sample (1:100 or 1:40 for whole tissue and MEO respectively) of RNA:10 mM TrisCl (pH 7.5) at 260 nm and 280 nm. Pure RNA has an absorbance ratio of 1.9 – 2.1. Total RNA was stored at -80°C until analysis.

3.5. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

The relative expression of mammary epithelial transporters was determined by quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) on a Cephied SmartCycler® platform (Cephied, Sunnyvale, CA) with the use of one-step Quantitect SYBR Green RT-PCR kits and reagents. Reactions were performed in a final volume of 25 μL as per Table 3.2.

TABLE 3.2 – QRT-PCR master mix reaction components for use on the Cephied SmartCycler®. Master mix provides a final concentration of 2.5 mM $MgCl_2$. Adapted from Qiagen (http://www.qiagen.com).

Component	Volume/Reaction	Final Concentration
2x Quantitect SYBR Green	12.5 μL	1x
Quantitect RT Mix	0.25 μL	-
Left Primer	2 μL	1 μM
Right Primer	2 μL	1 μM
RNase-free Water	6.25 μL	-
Template RNA	2 μL	≤ 500 ng
Total Volume	**25 μL**	**-**

Validation and optimization of the most favorable annealing temperature, which resulted in the highest primer efficiency (E) was performed (Equation 3.2) using control tissues from adult male Sprague-Dawley rats as positive control tissues where applicable (Table 3.3).

$$Slope(C_T) = -1\frac{1}{\log E} \qquad [3.2]$$

The optimal annealing temperature produces a single specific melt peak, which is not identified in a negative control (a blank sample containing no RNA template). QRT-PCR products were verified by horizontal 2% (w/v) agarose gel electrophoresis for a single band at the specified amplicon size correlating to the single, specific product melt peak. Primer efficiency was calculated (as per Equation 3.2) from the slope of a 3-point standard curve using serial dilutions of control RNA where a slope range of -2.8 to -3.5 and consequently efficiencies of 1.8 to 2.2 were considered as optimal. The reactions were quantified following determination of the threshold cycle (C_T; the amplification cycle when PCR products are first detected above baseline fluorescence) and fluorescence was measured from the intercalation of SYBR green dye into the double-

stranded product after the primer elongation phase (step 4 & 5; Figure 3.2). A non-template negative control was incorporated into all analysis runs.

The QRT-PCR protocol consisted of reverse transcription (1 cycle at 50°C for 30 minutes), PCR initial activation step (1 cycle at 95°C for 15 minutes), three-step thermal-cycling (50 cycles; denaturing at 94°C for 15s, annealing at 57°C-58°C for 30s, and extension at 72°C for 30s), and a melt curve analysis from 65°C-95°C at 0.5°C/s (Figure 3.2).

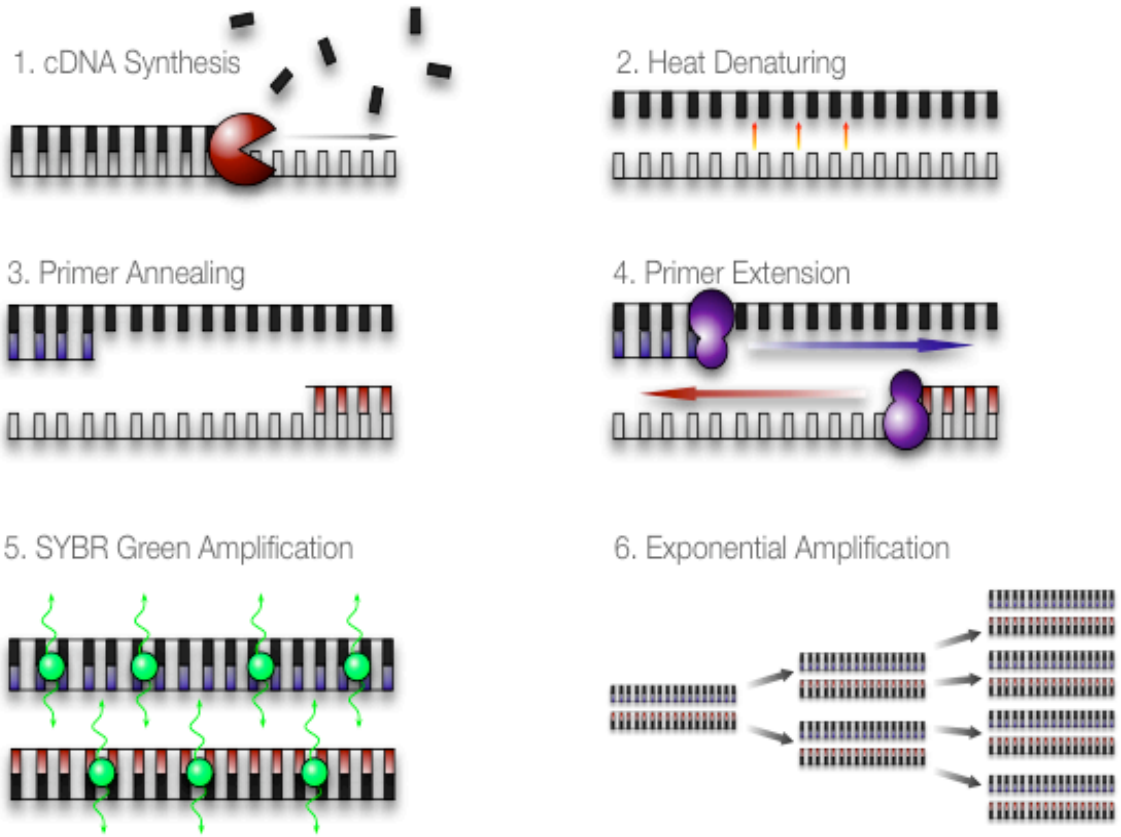


FIGURE 3.2 – Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) and intercalation of SYBR green into double stranded DNA during the elongation phase.

Quantification of targets in the unknown samples were determined by interpolation from a 5-point standard curve constructed from serial dilutions of the appropriate male control tissue (where applicable; Table 3.3) which spanned 5-orders of

magnitude. All relative fluorescence results are expressed as relative fluorescence units normalized to total RNA[196-199].

3.6. Primer Design for QRT-PCR

Gene cDNA sequences for each respective transporter were obtained from the national center for biotechnology information Genebank (NCBI; http://www.ncbi.nih.gov) with primer sequences designed using Primer3 software (http://www.broad.mit.edu/cgi-bin/primer/primer3), a web-based primer design program. All primers were designed to be between 18-20 base pairs in length to accommodate the product size restrictions of the Cephied Smartcycler® QRT-PCR platform.

Table 3.3 – Primer optimizing conditions for mRNA expression analysis of transporters using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR).

Target Gene	Accession Number	Left Primer	Right Primer	Annealing Temperature	Primer Efficiency	Control Tissue
Mdr1a	NM_133401	gacggacaggacatcagg	ttctgtcccccactcagc	58°C	2.04	Ileum[200]
Mdr1b	NM_012623	ccagagtgggagacaaagg	gttctggatggtggacagg	58°C	1.87	Ileum[200]
Mrp1	NM_022281	ggtgggtgctgtttaccg	gctagggtgacttgcagagg	57°C	2.08	Duodenum[201]
Oct1	NM_012697	gtgtccggtgtgctaacagc	cactagccccactgtgaagg	58°C	1.91	Kidney[202]
Octn1	NM_022270	catggctgtgcagactgg	gcaccatgtagccgatgg	58°C	1.8	Kidney[202]
Octn2	NM_019269	ggcgcaaccacagtatcc	ggggctttccagtcatcc	58°C	1.90	Kidney[202]
Octn3	NM_019723	gacaccgtgaacctgagc	ccatccaggcagttctcc	58°C	1.85	Gonad[203]
Cnt1	NM_053863	cctggctgtactagccttcg	ccagcaactcagccactacc	58°C	2.09	Jejunum[204]
Cnt2	NM_031738	ctactacctgggccttgtgc	aggctcccaacactgtgc	58°C	1.97	Duodenum[204]
Cnt3	NM_080908	tggcctgaaacagagaaacc	gtcgaacatgcttccaaacc	58°C	2.00	Lung[204]

Continued…

Table 3.3 continued…

Ent1	NM_031684	gaaggagaggagccaagagg	gctggattccacctcagc	58°C	1.85	Lung[204]
Ent2	NM_031738	agcaccctcttcctcagtgg	cgaggagctcagctttgg	57°C	1.88	Gonads[204]
Ent3	NM_181639	tgtggggctctacctactgc	gggcggtgatgaagtagagg	58°C	1.93	Kidney[204]
Ncbt1	NM_017315	caggcgttcctgaagtgg	ctcactgttggcgtgtgc	58°C	2.10	Liver[91]
Ncbt2	NM_017316	aacaccatcccaggaacc	cgcctgttctcactcttcc	58°C	2.20	Liver[91]
Pept1	NM_057121	aaccactggctggactgg	ttcaccgtctgcatctgg	58°C	1.89	Ileum[205]
Pept2	NM_031672	cctggttacagcagcagagg	caggccactgaactgagc	58°C	1.84	Kidney[205]
Mtx1	AF173642	ttcagcctgagcctctcc	agacgcagctgtggttgc	58°C	1.95	Duodenum[206]
Dmt1	NM_013173	aggagctagggcatgtgg	ccaccgctggtatcttcg	58°C	1.92	Liver[71]
β-casein	NM_017120	tgcaagggagaaggatgc	tgggactgcaagagatgg	58°C	1.92	Mammary[207]
β-actin	NM_031144	agcgtggctacagcttcacc	tgccacaggattccataccc	58°C	1.93	Liver
GAPDH	NM_017008	acgacccttcattgacc	tgaagacgccagtagactcc	58°C	1.98	Liver

3.7. Primary Mammary Epithelial Organoid (MEO) Cell Culture System

Mammary epithelial organoids were isolated from whole mammary gland tissue removed from female Sprague-Dawley rats at early lactation stage (day 1) and mid lactation stage (day 10) as described above (with minor modifications) and cultured as per the method of Darcy *et al.*[185] with culture media modifications as per Debnath *et al.*[193]. Following the initial filtration step (through 530 μm filter), two 1 mL aliquots of MEO suspension were removed and transferred to sterile 15 mL polypropylene centrifugation tubes for nuclei isolation and quantification. During the 4 h selective adherence incubation, aliquoted MEO were passed 10-15 times through a 1 mL syringe bearing a 22-guage needle and incubated with 7 mL of 0.1M citric acid solution (Table 3.1) for 90 min at 37°C in a humidified CO_2 incubator. The nuclei and residual cells were then collected using a 10 min 2560 × *g* centrifugation at 4°C and the pellet resuspended in 1 mL of ice-cold nuclei preparation buffer (Table 3.1). The nuclei suspension was then passed 10-15 times through a 3 mL syringe equipped with a 25-guage needle to break up and remaining intact cells. Using a hematocytometer and the resulting nuclei suspension, the number of nuclei present (per milliliter) was quantified using 10 μL of a mixture of 90 μL nuclei suspension and 10 μL trypan blue solution (Table 3.1).

Following the 4h selective adherence incubation, the nonadherent MEO recovered after incubation were transferred to a sterile 50 mL polypropylene centrifuge tube and an aliquot of MEO suspension corresponding to 1.6×10^7 cells was removed and placed in a sterile 15 mL polypropylene centrifuge tube. The remaining cells were suspended in cryopreservation medium (Table 3.1) at a density of 2-5 $\times 10^7$ cells/mL and stored at -80°C. The separated aliquot was pelleted by a 10 min 500 × *g* centrifugation at 4°C and resuspended in 4 mL of primary cell culture media containing 2% Matrigel™ (4×10^6 cells/mL). Three milliliters of this solution (1.2×10^7 cells) was removed and plated on 35 mm Matrigel™ coated plates (Figure 3.3) at 37°C in a humidified CO_2 incubator for 4 days or 8 days (primary culture media was changed every 3.5-4 days of plating).

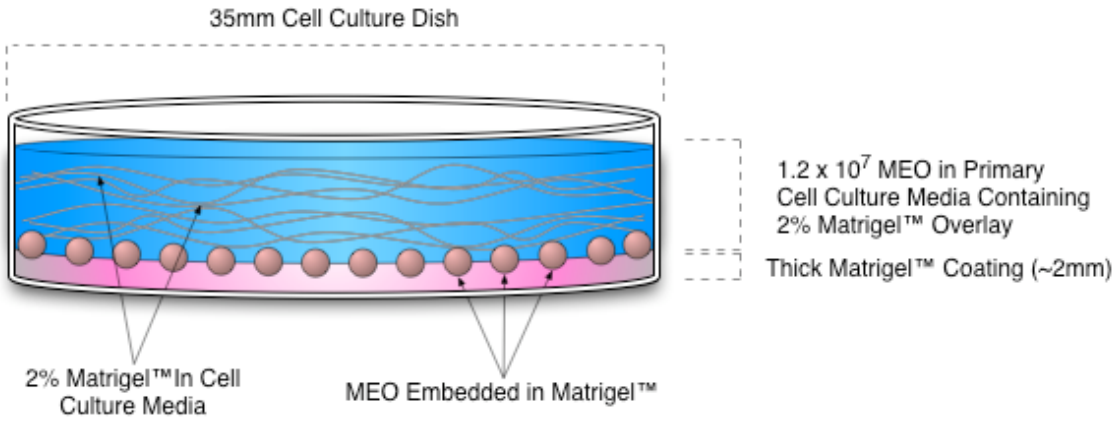


FIGURE 3.3 – Schematic illustration of the MEO plating conditions. MEO were isolated from female Sprague-Dawley rats during early (day 1) and late (day 10) lactation and cultured in 35 mm Matrigel™-coated cell culture plates in a defined serum-free medium containing a 2% Matrigel™ overlay. See text for more detailed instructions.

On the appropriate harvesting day (day 4 or day 8), MEO were harvested using a cell recovery solution (MatriSperse™) as per the manufacturer's protocol. Briefly, primary culture media was removed from each plate and the cells were washed 3 times with ice-cold PBS. Two millilitres of cell recovery solution was placed in each dish, and the dish contents gently scraped into a sterile 50 mL polypropylene centrifugation tube, inverted a few times and left on ice for ~1.5 h (or until Matrigel™ was completely dissolved and cells had settled to the bottom of the tube). The cells were collected with a 5 minute 250 × *g* centrifugation at 4°C and washed twice in ice-cold PBS. The final cell pellet was subjected to RNA extraction.

Total RNA was isolated using RNeasy Midi isolation kits as per the manufacturer's directions. The recovered cell pellet from each dish (~ 1.2×10^7 cells) was homogenized in 3.5 mL of lysis buffer using a Polytron tissue homogenizer. A volume ($2.5 \cdot V_1$; where V_1 is the initial volume of lysis buffer) of 100% ethanol was added to the homogenate and the sample was vigorously mixed to precipitate nucleic acids and proteins. The homogenate was applied to the Midi column and cellular contaminants (including genomic DNA) were washed away using a series of centrifugation steps and various buffers. The purified RNA sample was eluted from the column using RNase-free water (2 × 150 µL) and quantified as described above. Total RNA was stored at -80°C until analysis.

TABLE 3.4 – Detailed preparation instructions for primary cell culture medium for the plating of mammary epithelial organoids (MEO) isolated from whole mammary gland tissue. Adapted from Darcy *et al*.[185]

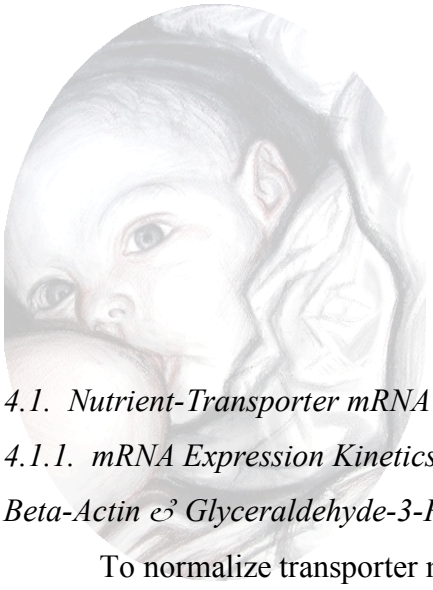
Ingredient	Solvent	Stock Conc.	Final Conc.
DMEM-F12	Liquid Reagent	1x	1x
Fatty-acid-free BSA	DMEM-F12	10 mg/mL	1 mg/mL
Gentamicin	Liquid Reagent	50 mg/mL	50 μg/mL
Bovine insulin	0.01N HCl	10 mg/mL	10 μg/mL
Human apo-transferrin	DMEM-F12	5 mg/mL	5 μg/mL
Ovine prolactin	0.01N NH_4OH	1 mg/mL	1 μg/mL
Progesterone	100% EtOH[a]	1 mg/mL	1 μg/mL
Hydrocortisone	100% EtOH[a]	1 mg/mL	1 μg/mL
Ascorbic acid	DMEM-F12	0.88 mg/mL	0.88 μg/mL
Mouse EGF	Liquid Reagent	1 mg/mL	10 ng/mL
Matrigel™	Liquid Reagent	1x	2% (v/v)

[a]EtOH – ethanol

3.8. Descriptive Statistics

QRT-PCR data for whole mammary gland tissue is expressed as the mean ± S.D. of 4 independent analyses for each group.

Due to tissue availability, MEO tissue was pooled at the organoid stage prior to RNA isolation. Therefore, QRT-PCR data for MEO is expressed as the mean of duplicate analyses of pooled MEO (n = 4/group) tissue and data will give no representation of standard deviation.



- IV -

Results

4.1. Nutrient-Transporter mRNA Temporal Expression Kinetics

4.1.1. mRNA Expression Kinetics of Potential "Housekeeping Gene" Candidates Beta-Actin & Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

To normalize transporter mRNA expression levels, the mRNA expression levels of two housekeeping genes, β-actin and GAPDH, were assessed in all stages of gestation, lactation and involution evaluated for the transporters. Figure 4.1 and 4.2 indicate that mRNA expression levels of both housekeeping genes do not remain constant with stage of mammary gland differentiation, although GAPDH tends to show more consistent expression than β-actin.

β-Actin Expression in Whole Mammary Gland Tissue

The message encoding β-actin in whole mammary tissue is relatively stable from gestation stages into the onset of lactation. However from mid lactation and for the remainder of the lactation period β-actin decreases in expression levels to less than 20% of levels seen at day 15 of gestation. With involution mRNA levels increase to exceed those seen in gestation (G15) by ~40% (Figure 4.1).

β-Actin Expression in Mammary Epithelial Organoids

β-actin expression in MEO is higher than levels in whole mammary gland tissue preparations and follows a slightly different temporal expression pattern from the whole tissue. β-actin expression decreases markedly from virgin by ~62% and ~70% at days

15 and 20 of gestation, respectively. Expression levels increase during early lactation, although they remain much lower than virgin levels (~55% of virgin levels). β-Actin mRNA levels steadily decline through lactation with reductions of ~18%, ~50%, and ~74% at days 3, 7, and 10 of lactation, respectively, relative to lactation day 1. By day 18 of lactation, mRNA levels of β-actin are <10% of lactation day 1 expression levels. At involution, mRNA levels slightly recover to ~50% of the levels seen at day 1 of lactation (Figure 4.1).

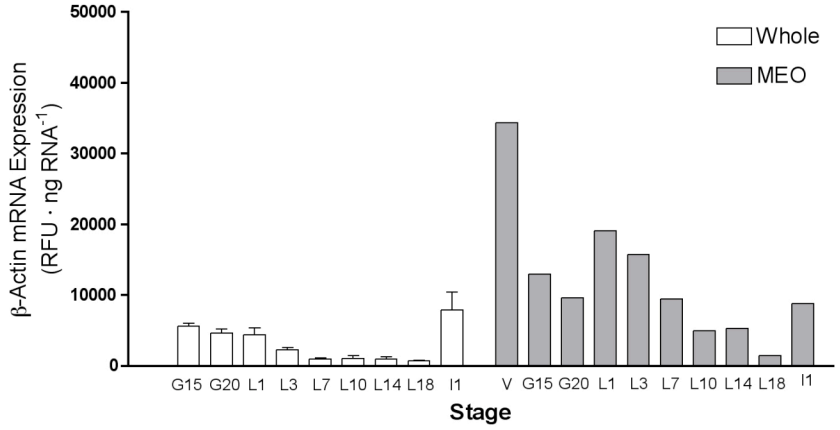


FIGURE 4.1 - mRNA expression analysis of β-actin in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, closed bars). Total mRNA was isolated from whole mammary tissue and isolated mammary epithelial organoids from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

GAPDH Protein Expression in Mammary Epithelial Organoids

GAPDH was evaluated only in MEO. (A consistent expression of GAPDH in MEO would have warranted further investigation into its expression in a whole tissue preparation.) GAPDH mRNA expression in MEO drops markedly from virgin levels by day 15 and 20 of gestation by ~70% and ~85%, respectively. GADPH mRNA increases by ~85% and ~110% at day 3 and day 7 of lactation, respectively, but decline thereafter reaching levels that are ~50% higher than lactation day 1 expression values. Involution

mRNA levels increase to reach ~50% of the expression levels seen in the virgin tissues (Figure 4.2).

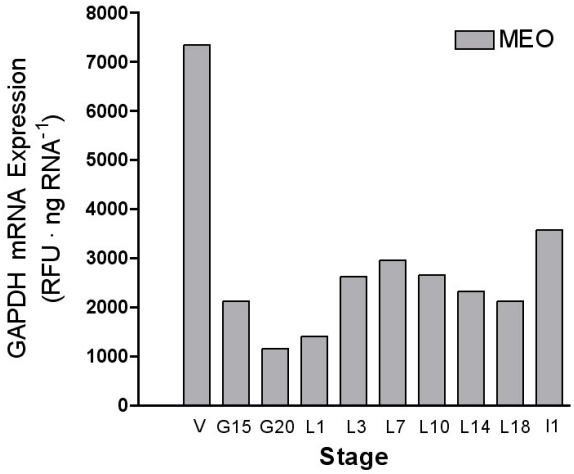


FIGURE 4.2 - mRNA expression analysis of GAPDH in isolated mammary epithelial organoids (MEO). Total mRNA was isolated from isolated mammary epithelial organoids from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

4.1.2. mRNA Expression Kinetics of ATP-Binding Cassette Transporter Super Family Throughout Pregnancy & Lactation

4.1.2.1. The Multi-Drug Resistance Proteins (Mdr; P-Glycoprotein)

Mdr Protein Expression in Whole Mammary Gland Tissue

Mdr1a mRNA expression levels in whole mammary tissue is markedly reduced throughout pregnancy and the whole lactation period. mRNA levels of Mdr1a at the onset of lactation drop to reach levels less than 25% of the levels seen at day 15 of gestation, with further reductions to <10% for the remainder of lactation. mRNA expression levels begin to recover with early involution(~80% of those seen at the onset of lactation) (Figure 4.3A).

Mdr1b expression is nearly diminished with the onset of lactogenesis with levels throughout the entire lactation period expressed at less than 5% of the expression level seen at day 15 of gestation. Similar to Mdr1a, Mdr1b levels at involution recover slightly and reach levels similar to those seen at day 20 of gestation (Figure 4.3B).

Mdr Protein Expression in Mammary Epithelial Organoids

Although Mdr1a and Mdr1b mRNA expression levels in MEO is markedly higher than whole tissue preparations, the pattern of expression is similar. Mdr1a mRNA levels drop ~68% and ~72% from the quiescent state (i.e. virgin) by day 15 and day 20 of gestation, respectively. These levels continue to decrease into early stage lactation (day 1) with mRNA expression levels reduced to ~10% of that seen in virgin tissues. Despite a slight recovery by day 7 lactation (+ 177% relative to day 1 lactation), the mRNA levels of Mdr1a remain low for the remainder of the lactation period (> 90% reduction relative to virgin). These levels show another small recovery with the onset of involution with mRNA levels increasing 9% relative to day 1 of lactation (Figure 4.3A).

Mdr1b expression levels and pattern are highly similar to those of Mdr1a throughout the lactation period and involution. mRNA expression levels for Mdr1b drop during pregnancy reaching levels equivalent to ~45% and 35% of the expression seen in the quiescent state and these levels continue to drop with the onset of lactation, where early stages of lactation are also markedly lower than those levels seen in virgin tissues (< 15% of the expression seen in virgin at lactation day 1 and 3). Mid-stage and late-stage lactation reflect a similar trend of decreasing mRNA expression levels. Despite a minor recovery of mRNA levels at day 7 of lactation, both mid- and late-stage lactation levels are less than 10% of those levels seen in the virgin animals. With involution mRNA levels increase to levels seen in early lactation stages (~ 15% of the virgin level; Figure 4.3B).

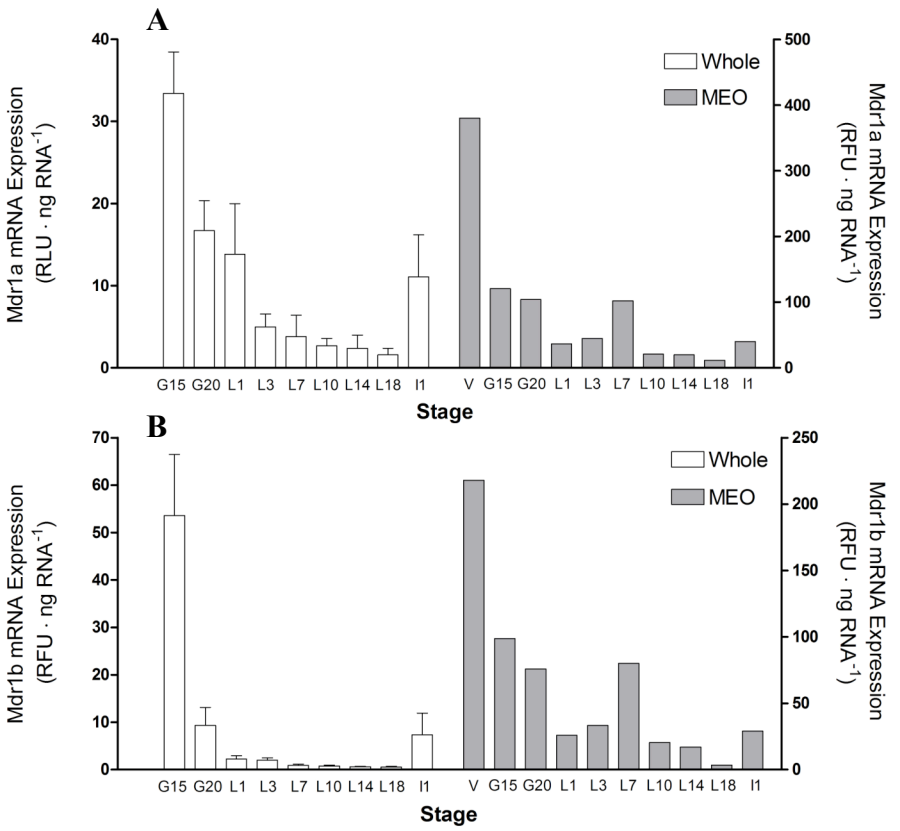


FIGURE 4.3 - mRNA expression analysis of the multi-drug resistance proteins, Mdr1a (A) and Mdr1b (B), in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue and isolated mammary epithelial organoids from virgin (V) female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate). MEO expression data are represented by the right y-axis.

4.1.2.2. The Multi-Drug Resistance-Like Proteins (Mrp)

Mrp1 Protein Expression in Whole Mammary Gland Tissue

Mrp1 mRNA expression remains similar during gestation and day 1 of lactation, however begin to drop thereafter and reach levels at mid lactation less than 25% of those seen at lactation day 1. Levels remain constant for the remainder of the lactation period. Involution results in enhanced mRNA expression to levels ~80% of those seen during gestation (Figure 4.4).

Mrp1 Protein Expression in Mammary Epithelial Organoids

Mrp1 mRNA expression in MEO is similar in magnitude and temporal expression pattern to those seen in whole mammary tissue preparations. Mrp1 levels in MEO decrease by ~60% at day 15 of gestation relative to virgin, which was further reduced to ~30% of that seen in virgin tissues by day 20 of gestation. During early lactation, mRNA levels remained relatively constant (~ 30% of virgin values) until mid-lactation where expression levels were reduced to less than 10% of that seen in virgin, and continued to decline until involution. The onset of involution showed a marked recovery from later stage lactation (day 18) as expression levels rose to similar levels seen in the early stages of lactation (~ 30% of those seen in virgin tissues; Figure 4.4).

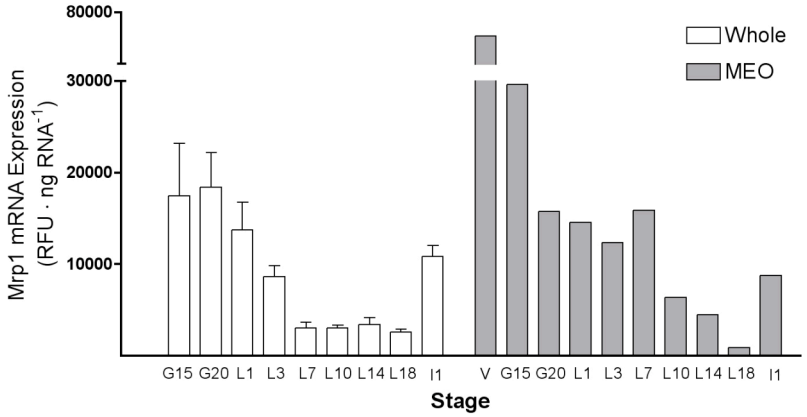


FIGURE 4.4 - mRNA expression analysis of the multi-drug resistance-like protein, Mrp1, in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue and isolated mammary epithelial organoids from virgin (V) female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

4.1.3. mRNA Expression Kinetics of Solute Carrier Transporter Super Family Throughout Pregnancy & Lactation

4.1.3.1. The Organic Cation Transporter Family (Oct)

Oct1 Transport Protein Expression in Whole Mammary Gland Tissue

Oct1 mRNA expression remains low throughout the late stages of pregnancy and early lactation. Thereafter, mRNA levels increase steadily and by day 14 of lactation are more than 1700% greater than those seen at lactation day 1. Levels thereafter decline into involution where expression levels are similar to those seen at day 15 of gestation (Figure 4.5).

Oct1 Transport Protein Expression in Mammary Epithelial Organoids (MEO)

The message for Oct1 in MEO is markedly higher in whole tissue preparations compared to isolated MEO. Oct1 mRNA expression levels in MEO was decreased to ~35% and ~40% of that seen in virgin tissue by day 15 and day 20 of gestation, respectively. The onset of lactation was characterized by an increase in mRNA levels exceeding the virgin level (~35% at lactation day 1), which continued to increase and peak (~110% relative to virgin tissues) by mid lactation. Late lactation showed a steady decline in mRNA levels which, by day 18, had reached a level similar to those seen in late gestation with levels of ~65%, ~50%, and ~30% of those seen in early lactation (day 1) for lactation days 10, 14, and 18, respectively. With involution, mRNA levels returned to similar expression levels as observed in the virgin mammary gland. Despite the large relative changes, Oct1 in MEO is expressed at very low levels (less than 0.35 RFU · ng RNA^{-1}) throughout the entire lactation period (Figure 4.5).

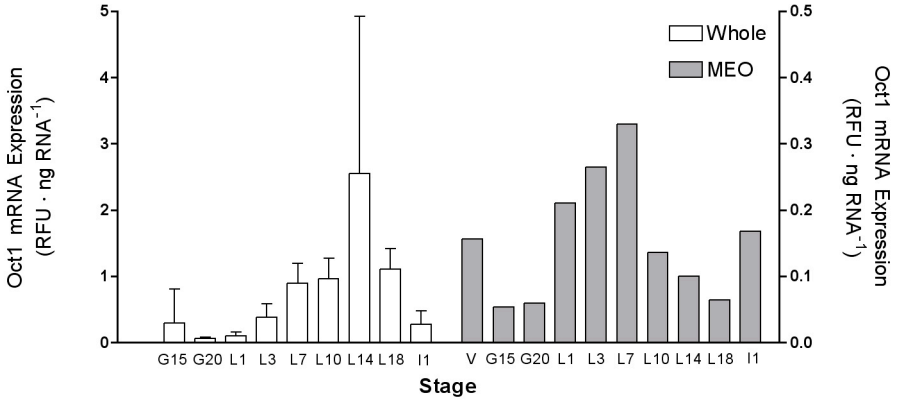


FIGURE 4.5 - mRNA expression analysis of the organic cation transporter, Oct1, in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue and isolated mammary epithelial organoids from virgin (V) female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate). MEO expression data are represented by the right y-axis.

4.1.3.2. Organic Cation/Carnitine Transporters (Octn)

Octn Transport Proteins in Whole Mammary Gland Tissue

The organic cation/carnitine transporters 1 and 2 are the most dominantly expressed transporters of the Octn transporter family in gestation periods. During lactation, Octn1 is expressed at higher levels than Octn2. Octn1 mRNA expression remains nearly constant from gestation into the initial stages of lactation. By mid lactation (days 3, 7, and 10) levels increase reaching levels ~300%, ~255%, and ~208% greater than lactation day 1, respectively. mRNA expression in later stages of lactation decline to reach levels nearly equivalent to early lactation (L1). At involution mRNA levels are higher than day 1 of lactation by ~270% (Figure 4.6A).

Octn2 mRNA levels decline steadily from late gestation throughout the lactation period. Early lactation stages (L1 and L3) are marked by a decrease in mRNA levels from gestation by ~50% for both days. mRNA expression continues to decline throughout the remainder of lactation. By day 7, levels drop to reach ~60% of lactation

day 1 values and by late stage lactation and involution values less than ~25% of those seen at lactation day 1 was observed (Figure 4.6B).

mRNA levels for the third member of the Octn family, Octn3 follow a similar expression pattern as Octn1. Levels drop between late gestation and early lactation (L1) by ~40%, and then begin to increase until mid lactation. Message levels reach ~130%, ~120%, and 100% of those seen in early lactation (L1) for days 3, 7, and 10 of lactation, respectively, and drop further into late stage lactation (L14 and L18; less than 60% of L1 values). (Figure 4.6C).

Octn Transport Proteins in Mammary Epithelial Organoids (MEO)

The expression of Octn1 and Octn2 in MEO is similar to the expression pattern and levels seen in whole mammary gland preparations, however Octn3 mRNA is markedly different in MEO, both in expression levels and expression pattern relative to whole tissue preparations.

The Octn1 shows the highest expression of all members of the Octn family in the quiescent state with expression levels higher than Octn2 and Octn3 by ~950% and ~850%, respectively. However, while Octn1 and Octn2 show a decrease in expression throughout the lactation period, Octn3 shows a continual increase through most of the lactation period to be the most dominantly expressed member of the Octn transporter family during lactation (Figure 4.6).

Relative to virgin tissues, the mRNA expression for Octn1 is markedly lower for all stages of gestation, lactation, and involution. Gestation levels drop to ~6% and ~5% of the expression level seen in virgin tissues at day 15 and day 20 of gestation, respectively. The levels continue to remain low during early lactation (days 1 and 3, < 5% of virgin levels). A marginal recovery at lactation day 7 relative to day 1 lactation (+ ~220%) was observed, however expression remains less than 15% of the levels seen in the virgin state. Late lactation is characterized by a gradual decline in expression levels, which by day 18 of lactation reach levels that are less than 1% of those seen in virgin. Involution results in enhanced expression to levels similar to those seen in day 7 lactation at ~15% of the expression levels seen in virgin tissues (Figure 4.6A).

Octn2 mRNA expression levels are characterized by a steady decline from the virgin state throughout pregnancy with levels at day 15 and day 20 of lactation

reflecting a ~20% and ~40% decrease in expression from virgin, respectively. During early lactation levels continue to decline to reach ~50% of those seen in virgin mammary gland. Octn2 mRNA levels rapidly recover at day 3 of lactation to reach levels similar to those seen in virgin tissue (~ 90%) followed by a similar rate of decline as seen in late-stage pregnancy with values at day 7, 10, 14, and 18 of lactation equivalent to ~80%, ~55%, ~45%, and ~15% of the mRNA levels seen in virgin tissues. With involution mRNA levels increase to slightly higher levels than those seen at day 1 of lactation (~ 11%; Figure 4.6B).

Octn3 levels decrease with the onset of pregnancy, with levels in mid and late gestation expressed at ~80% and ~30% of the levels seen in the quiescent state, respectively. With the onset of lactation a consistent increase in Octn3 mRNA levels is observed and by mid-lactation stages (day 7 and 10) mRNA levels reach ~150% and ~280% relative to day 1 lactation and by late-lactation stages expression is ~380% and ~400% at day 14 and 18 of lactation relative to day 1 lactation, respectively. With involution further increases in Octn3 mRNA levels are observed and reach levels greater than 1200% relative to day 1 lactation (Figure 4.6C).

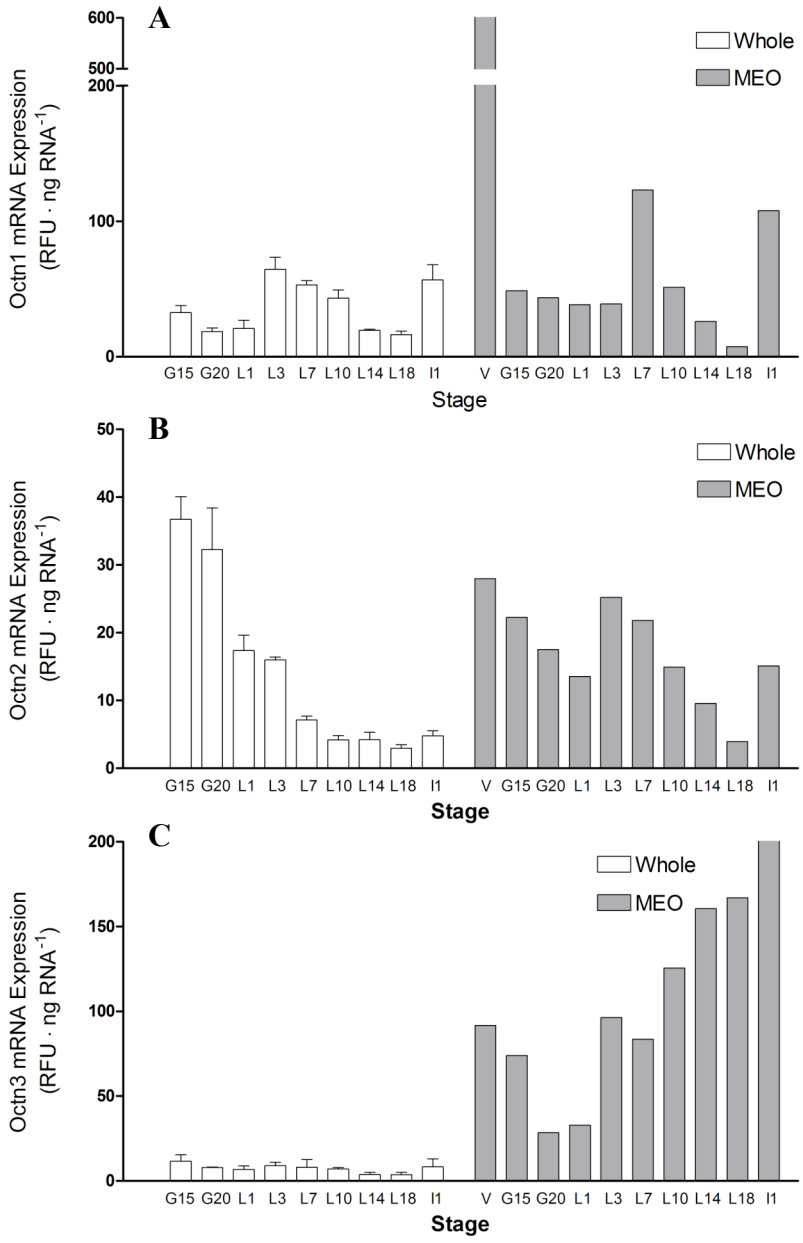


FIGURE 4.6 - mRNA expression analysis of the organic cation/carnitine transporters, (A) Octn1, (B) Octn2, ad (C) Octn3 in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue (Whole) and isolated mammary epithelial organoids (MEO) from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

4.1.3.3. The Equilibrative Nucleoside Transporter Family (Ent)

Ent Transport Protein Expression in Whole Mammary Gland Tissue

Equilibrative nucleoside transporters 2 and 3 are the most abundant of the Ent transporters during gestation periods (greater than 200% of Ent1 expression), however with the onset of lactation (L1 and L3), Ent2 and Ent3 values drop markedly and Ent1 becomes the most abundantly expressed protein (greater than 240% of levels seen for both Ent2 and Ent3). Late stage lactation (L10, L14, and L18) shows a recovery of Ent3 mRNA as Ent2 levels remain low and Ent1 levels drop and Ent3 now becomes most abundant during late stages of lactation (greater than 240% and 460% of Ent1 and Ent2, respectively; Figure 4.7).

The message for Ent1 increased markedly during early lactation to reach levels greater than 220% and 165% of levels seen at day 15 of gestation for lactation day 1 and 3, respectively. mRNA levels then drop into mid lactation and late lactation as the message for days 7, 10, 14, and 18 of lactation is less than 20% of lactation day 1 levels. With involution mRNA levels increase to slightly higher (15%) levels than day 15 of gestation (Figure 4.7A).

Ent2 mRNA decreases steadily throughout the lactation period. Late gestation levels drop by ~45% from day 15 of gestation and continue to drop into early lactation (L1) by an additional 10%. For the remainder of lactation Ent2 message drop to levels less than 50% relative to day 1 for days 3 and 7 of lactation and to less than 20% of L1 values for days 10, 14, and 18 of lactation. With involution mRNA levels increase to reach ~50% of those seen at day 1 of lactation (Figure 4.7B).

Ent3 mRNA decreases from gestation into early lactation where days 1 and 3 are ~30% of those seen at day 15 of gestation. mRNA expression continues to decline into day 7 of lactation (less than 15% of G15 values), however mRNA recovered for the later stages of lactation to exceed day 1 values by ~40% and ~20% for lactation days 14 and 18, respectively. The recovery continues into involution to reach levels greater than 114% of the message at day 1 of lactation (Figure 4.7C).

Ent Transport Protein Expression in Mammary Epithelial Organoids

The expression pattern and levels for both Ent1 and Ent2 are similar between MEO and whole mammary tissue preparations. However for Ent3, despite sharing some

similarities in expression level, the expression pattern is markedly different in MEO than in whole tissue preparations. Equilibrative nucleoside transporter 1 (Ent1), the highest expressed member of the Ent family, is expressed at levels greater than 450% and 350% of the expression levels of Ent2 and Ent3 during early lactation (day 1 and 3), respectively. However, mRNA levels by mid lactation and thereafter remains similar between all members of the Ent family (Figure 4.7).

Ent1 mRNA expression levels during mid-stage gestation (day 15) vary slightly from virgin tissue (~117% relative to the virgin state). However, by day 20 of gestation levels return to nearly equal those of the quiescent tissue. Early stages of lactation demonstrate a marked increase in Ent1 expression with increases of nearly 500% relative to the virgin tissues in early lactation (~511% and ~496% at lactation day 1 and day 3 respectively). There is a marked decline in mRNA expression during mid- and late stage lactation with levels at day 7 lactation dropping to ~38% of the levels seen at day 1 with a continued decline to less than 5% of day 1 levels by lactation day 18. With involution mRNA levels recover and reach ~243% higher levels than those seen in the quiescent state (Figure 4.7A).

Expression of the second member of the Ent family (Ent2) is markedly reduced throughout all stages of gestation, lactation, and involution relative to virgin tissues. Mid- and late stage pregnancy show a large decrease in expression of ~78% and ~90%, respectively, compared to virgin tissues. This decline in mRNA expression stays relatively constant throughout lactation and involution with all stages expressing mRNA levels less than 12% of those values seen in the virgin state (Figure 4.7B).

Similar to Ent2, the mRNA expression of Ent3 is markedly reduced throughout all stages of gestation, lactation, and involution relative to the quiescent state. All mRNA expression levels throughout these stages are expressed at less than 13% of those values seen with virgin tissues, with levels in the late stages of lactation (day 14 and day 18) further declining to less than 7% of virgin values (Figure 4.7C).

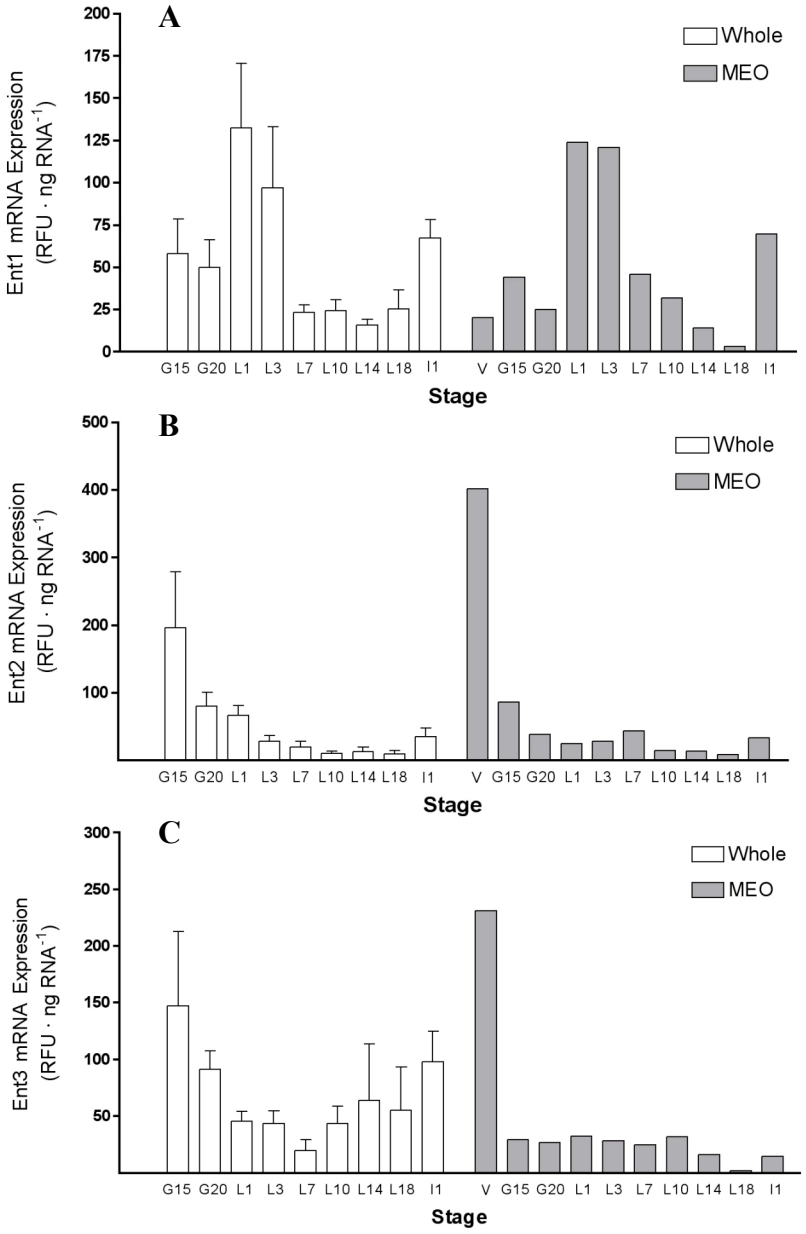


FIGURE 4.7 - mRNA expression analysis of the equilibrative nucleoside transporters; (A) Ent1 (B) Ent2 and (C) Ent3 in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue (Whole) and isolated mammary epithelial organoids (MEO) from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate). MEO expression data for Ent3 are represented by the right y-axis.

4.1.3.4. The Concentrative Nucleoside Transporter Family (Cnt)

Cnt Transport Protein Expression in Whole Mammary Gland Tissue

Concentrative nucleoside transporter member 3 (Cnt3) is the highest expressed member of the Cnt family throughout all observed stages of gestation, lactation, and involution. Message levels of Cnt3 exceed Cnt1 and Cnt2 mRNA by an average of more than 4,000-fold and 360-fold, respectively, for all stages analyzed (Figure 4.8).

Cnt1 mRNA drops into late pregnancy to reach levels at day 20 of gestation of ~65% of mid pregnancy values (G15). Early lactation shows a small increase in Cnt1 mRNA to reach levels at day 1 and 3 of lactation slightly higher than late pregnancy values (~82% and ~75% of G15 levels) followed by an additional increase into mid lactation stages (~120% and ~127% of G15 levels). In late lactation expression of Cnt1 decreases to reach levels similar to early lactation stages (~99% and ~80% of L1 expression). With involution message levels increase to exceed mid pregnancy levels by ~14%. Despite the fluctuation of Cnt1 mRNA, the range over which this fluctuation occurs is very small (5.6 – 11.2 RFU · ng RNA^{-1}) and can be considered as negligible (Figure 4.8A).

The message for Cnt2 is increased by ~250% and ~800% from mid gestation (G15) to late gestation (G20) and lactation day 1, respectively. Following the peak at the onset of lactation, Cnt2 mRNA levels begin to steadily decline throughout the remainder of the lactation period to reach levels of ~65%, ~44%, ~34%, ~26%, ~13%, and ~9% of lactation day 1 values at days 3, 7, 10, 14, 18 of lactation and day 1 of involution, respectively (Figure 4.8B).

Cnt3 mRNA expression exhibits a similar rate of increase into early lactation as Cnt2. Levels at day 20 of gestation and day 1 of lactation are ~200% and ~875% greater than day 15 of gestation, respectively. Thereafter, Cnt3 mRNA decreases slightly to reach ~70% of lactation day 1 values by day 3 of lactation where levels remain relatively constant for the remainder of the lactation period. The message for Cnt3 nearly disappears with the onset of involution where it reaches levels less than 2% of lactation day 1 values (Figure 4.8C).

Cnt Transport Protein Expression in Mammary Epithelial Organoids

Only the second member of the Cnt family, Cnt2, shared a similar expression level and pattern between MEO and whole tissue preparations. The remaining two members, Cnt1 and Cnt3 both showed markedly different expression levels and temporal expression patterns between MEO and whole mammary gland tissue. During early lactation (day 1 and 3), Cnt3 is expressed at markedly higher levels than Cnt1 and Cnt2 exceeding them by greater than 20,000% and 5,500%, respectively. However during the later stages of lactation (days 14 and 18), Cnt1 mRNA then predominates as levels are expressed much higher than Cnt2 and Cnt3, exceeding them by greater than 500% and 240%, respectively (Figure 4.8).

Mid-stage pregnancy (day 15) showed a slight increase in Cnt1 mRNA levels (~28% from the virgin state), which dropped more than 60% from virgin tissues in late stage pregnancy (day 20). With onset of lactation a gradual increase in mRNA levels was observed until day 7 of lactation where expression remained relatively constant ranging from ~180%-270% of the relative expression seen at day 1. The onset of involution caused a marked increase of Cnt1 mRNA levels, which reached levels ~735% and ~1350% relative to those observed in virgin tissues and at day 1 lactation, respectively (Figure 4.8A).

Cnt2 mRNA expression decreased ~45% from virgin tissues by day 15 of pregnancy where it then began a steady incline. The mRNA expression levels increased to reach a value ~14% greater than virgin tissues by day 20 of pregnancy and then peaked at day 1 of lactation with an expression level that was ~56% greater than virgin levels. Thereafter, Cnt2 levels declined ~12% by day 3 lactation and ~60% by day 7 lactation. Expression levels continued to steadily decline after day 7 lactation and then plummeted to reach less than 5% of the expression seen at the initiation of lactation. With involution mRNA levels increased to reach similar levels as mid-pregnancy at ~55% of the expression level seen in the quiescent state (Figure 4.8B).

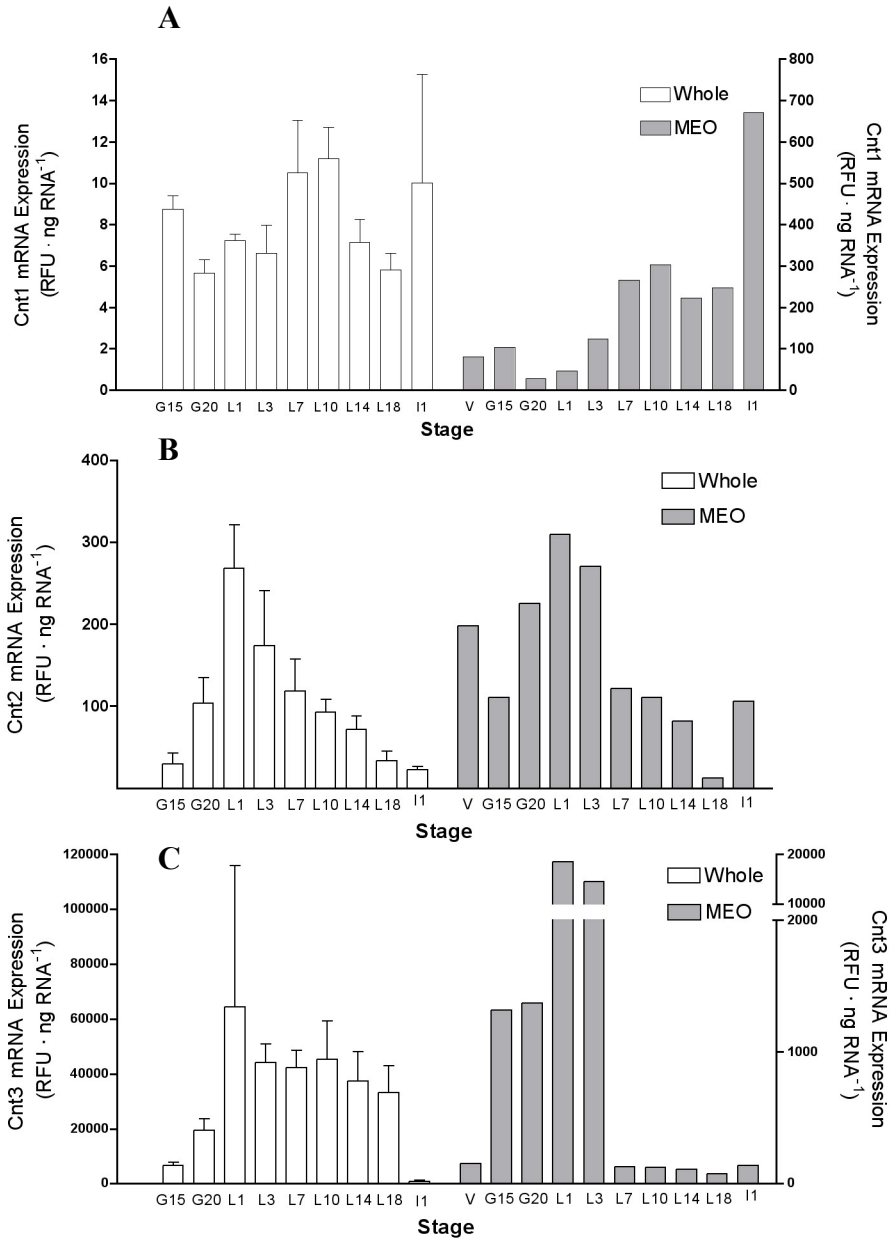


FIGURE 4.8 - mRNA expression analysis of the concentrative nucleoside transporters, (A) Cnt1 (B) Cnt2 and (C) Cnt3 in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue (Whole) and isolated mammary epithelial organoids (MEO) from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate). MEO expression data for Cnt1 and Cnt3 are represented by the right y-axis

The expression of Cnt3 mRNA rose dramatically during gestation to reach ~760% and ~795% relative to virgin levels at day 15 and day 20 of pregnancy, respectively. The onset of lactation sparked a tremendous increase in mRNA expression, with levels increasing more than 9,000% relative to the quiescent tissue during early lactation (day 1 and 3). However, by mid lactation (day 7), mRNA levels plummeted to ~16% lower than the levels seen in virgin tissues, and by late lactation Cnt3 expression remained at levels less than 1% of those seen at day 1 of lactation. With involution a slight recovery of mRNA levels was observed and reached ~90% of that seen in virgin tissues (Figure 4.8C).

4.1.3.5. The Nucleobase Transporter Family (Ncbt)

Ncbt Transporter Protein Expression in Whole Mammary Gland Tissue

Ncbt1 was expressed at lower levels than Ncbt2. Ncbt1 mRNA expression decreased from gestation into lactation to reach levels ~40% lower than day 15 of gestation by day 1 of lactation. Levels remained relatively constant throughout early and mid lactation, however the message was increased markedly during late lactation to reach levels at day 14 and 18 of lactation that exceeded lactation day 1 levels by ~130%, and ~430%, respectively. Involution caused a decrease in Ncbt1 mRNA to levels similar to lactation day 1 values (Figure 4.9A).

The second member of the nucleobase transporter family, Ncbt2, is expressed on average at levels over 300-fold higher for all analyzed time points than Ncbt1. Ncbt2 mRNA expression steadily decreased throughout pregnancy and early lactation reaching levels of ~12% of G15 levels by day 7 of lactation and remained relatively constant for the remainder of lactation (~20% of the message seen at day 1 of lactation). With involution Ncbt2 mRNA expression increased and approached levels observed during mid gestation (Figure 4.9B).

Ncbt Transporter Protein Expression in Mammary Epithelial Organoids

In MEO, Ncbt1 showed a markedly different temporal expression pattern and expression level than a whole tissue preparation. On the other hand Ncbt2 shared some similarities in expression levels between the two preparations but the relative expression pattern was different. Relative to other transporters, Ncbt1 was expressed at low levels throughout all stages of gestation, lactation, and involution in MEO. Expression levels

increased during gestation to reach ~278% of virgin levels by day 15 of gestation, with a slight drop by day 20 of gestation to a relative value of ~150%. Expression continued to increase during the early stages of lactation and remained relatively steady into day 7 of lactation (~270% relative to virgin). By day 10 lactation expression began to decline, with expression levels at day 10, 14, and 18 of lactation equivalent to ~68%, ~53%, and ~35% of those expression levels seen at the onset of lactation (day 1). Involution caused another slight recovery of Ncbt1 expression that exceeded the levels seen in virgin tissues by ~52% (Figure 4.9A).

The second member of the nucleobase transporter family (Ncbt2) was expressed at much higher levels than Ncbt1 (> 1000%). Expression levels markedly decreased during pregnancy with levels at day 15 and 20 of gestation only ~25% and ~17% of the expression seen in the virgin state. By day 1 of lactation, mRNA levels continued to decline representing ~9% of the expression level observed in virgin animals. At day 3 and day 7 of lactation mRNA levels increased ~45% and ~140% from day 1 lactation, respectively, to reach similar levels observed in mid-stage pregnancy. Following lactation day 7, mRNA levels began to decline with levels at day 10 and 14 similar to those seen at the onset of lactation (~10 greater and ~5% lower than lactation day 1 respectively) and by day 18, levels have dropped to ~35% of the levels seen at day 1 of lactation. Involution initiated a small recovery of mRNA levels that increased ~25% relative to lactation day 1 (Figure 4.9B).

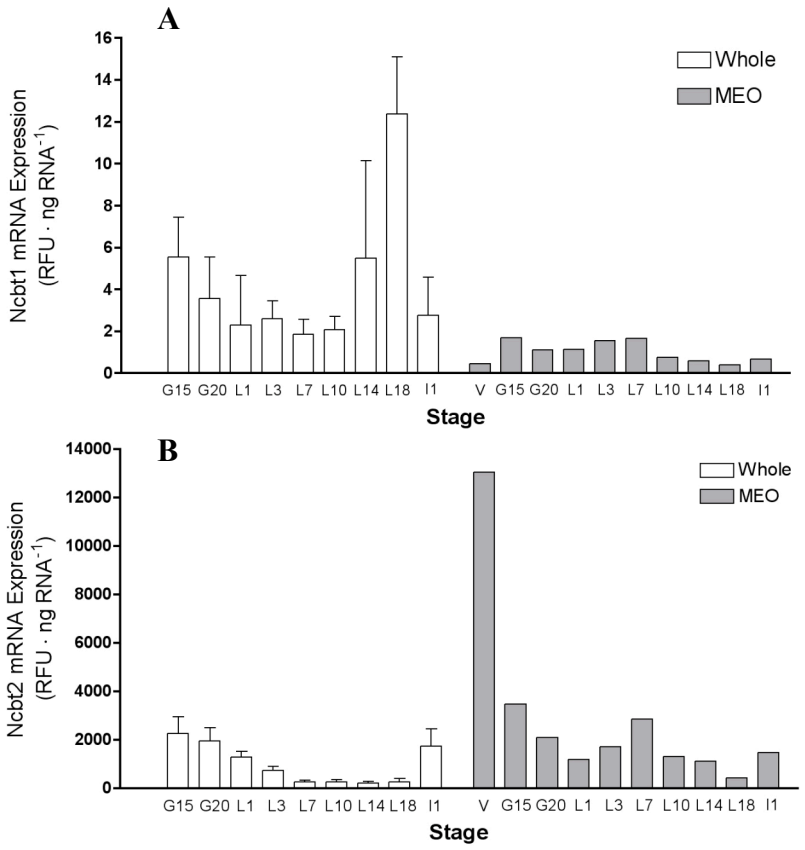


FIGURE 4.9 - mRNA expression analysis of the nucleobase transporters, (A) Ncbt1 and (B) Ncbt2 in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue (Whole) and isolated mammary epithelial organoids (MEO) from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

4.1.3.6. The Oligopeptide Transporter Family (Pept)

Pept Transport Protein Expression in Whole Mammary Gland Tissue

Pept1 expression in whole mammary gland tissue is sporadic and follows no distinct expression pattern. Expression remains relatively constant from gestation to the onset of lactation (day 1). Pept1 mRNA then quickly increases by ~210% from day 1 to day 3 of lactation followed by a drastic drop of signal by mid lactation with levels at day 7 and 10 expressed at ~18% and ~26% of lactation day 1 values respectively. A

recovery of mRNA at day 14 of lactation (~155% of L1) is followed by another drop in signal at day 18 (~27% of L1). Involution drives a spike in Pept1 mRNA to exceed levels seen at lactation day 1 by over 700%. Despite the large fluctuations in expression, the message for Pept1 is extremely low and expressed at levels less than 3 RFU · ng RNA^{-1} (Figure 4.10A).

The second member of the Pept family, Pept2, has more than 90-fold higher expression levels than Pept1 for all time periods measured. Pept2 mRNA levels decrease by ~30% from gestation day 15 to late stage gestation, which is followed by an immediate recovery at day 1 of lactation to levels similar to day 15 of gestation. During early lactation Pept2 message levels drop such that day 3 and 7 of lactation are ~60% and ~16% of levels seen at day 1 of lactation, respectively. Thereafter, Pept2 mRNA levels recover during mid and late lactation with levels at days 10, 14, and 18 of lactation demonstrating at ~23%, ~35%, and ~60% of lactation day 1 values, respectively. With involution expression levels decrease to < 30% of day 1 values (Figure 4.10B).

Pept Transport Protein Expression in Mammary Epithelial Organoids

The Pept transporters share certain similarities with whole tissue preparations in expression levels at certain stages of lactation, however the temporal expression pattern was markedly different between the two preparations. Pept1 mRNA in MEO was expressed at much lower levels than the second member of the oligopeptide transporter family (Pept2). At mid-gestational stage (day 15), mRNA levels decreased ~65% from virgin tissues which was quickly compensated in later-stage pregnancy (day 20) with marked recovery in mRNA levels that exceed those seen in the virgin animals (~150%). Expression once again decreased in early lactation, which by day 3 of lactation dropped below the low levels observed at day 15 of pregnancy (a decrease of ~75% relative to virgin). At day 7 of lactation mRNA reached levels ~110% greater than day 1 of lactation, which was followed in later stages of lactation by a gradual decline in mRNA levels equivalent to ~78% and ~27% higher levels as those seen at day 1 lactation for days 10 and 14 of lactation, respectively, and levels at day 18 decreasing below those seen at day 1 by ~46%. With involution mRNA levels increased to levels similar to

those seen by later-stage gestation (day 20) with levels that exceeded virgin tissues by ~150% (Figure 4.10A).

Pept2 expression is considerably higher (>6000%) than Pept1 in the early stages of lactation, however remains similar throughout the remainder of lactation. mRNA levels are greatly increased from virgin levels by day 15 and 20 of gestation exceeding virgin levels by ~570% and ~250 respectively. Early lactation caused another increase in mRNA levels, where levels at day 1 of lactation reached their highest expression (greater than 1000% of the levels seen in the virgin tissues), which decreased slightly (~23%) into day 3 of lactation. Mid and late lactation stages demonstrated a marked reduction in Pept2 mRNA levels, where all expression values for days 7, 10, 14, 18 of lactation, and day 1 of involution are all less than 8% of the expression seen at day 1 of lactation (Figure 4.10B).

4.1.3.7. The Methotrexate Carrier (Mtx)

Mtx1 Transport Protein Expression in Whole Mammary Gland Tissue

Mtx1 mRNA in whole mammary gland tissue is upregulated from day 15 of gestation to reach levels ~500% greater by day 20 of pregnancy. With the onset of lactation, Mtx1 mRNA levels decline such that by day 10 of lactation levels were similar to those seen at day 15 of gestation. This expression remained low and constant for the remainder of lactation. With involution Mtx1 mRNA reached levels similar to early lactation at more than 300% greater than day 15 of gestation (Figure 4.11).

Mtx1 Transport Protein Expression in Mammary Epithelial Organoids

Mtx1 in MEO exhibited a markedly different temporal expression pattern in MEO relative to whole mammary gland preparations, however in MEO, Mtx1 mRNA levels are expressed at a similar magnitude to whole mammary gland tissue. During pregnancy Mtx1 mRNA levels decline in MEO and by day 15 and 20 of gestation, levels are ~60% and ~40% of the expression seen in the virgin state, respectively. The onset of lactation was characterized by a gradual increase in mRNA expression levels, which peaked during mid lactation (~160% increase at day 7 relative to day 1 lactation) exceeding those levels seen in the virgin tissues by ~30%. In the remainder of the lactation period mRNA levels gradually declined to reach levels similar to day 1

lactation (~7% higher). With involution mRNA levels increased markedly to exceed virgin levels by ~26% (Figure 4.11).

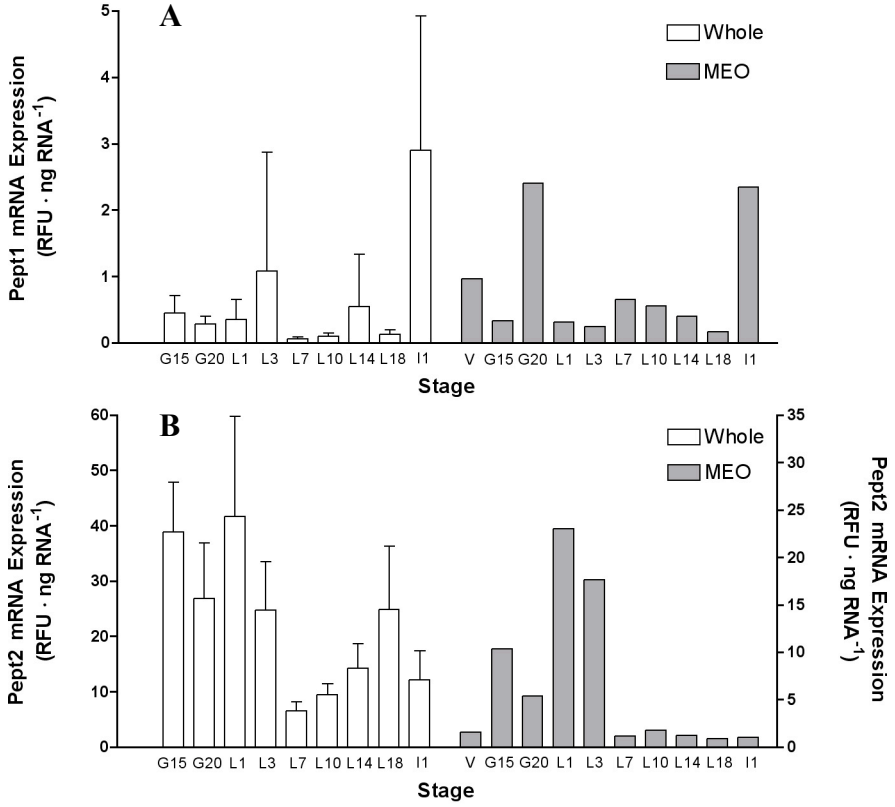


FIGURE 4.10 - mRNA expression analysis of the oligopeptide transporters, (A) Pept1 and (B) Pept2 in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue (Whole) and isolated mammary epithelial organoids (MEO) from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate). MEO expression data for Pept2 are represented by the right y-axis.

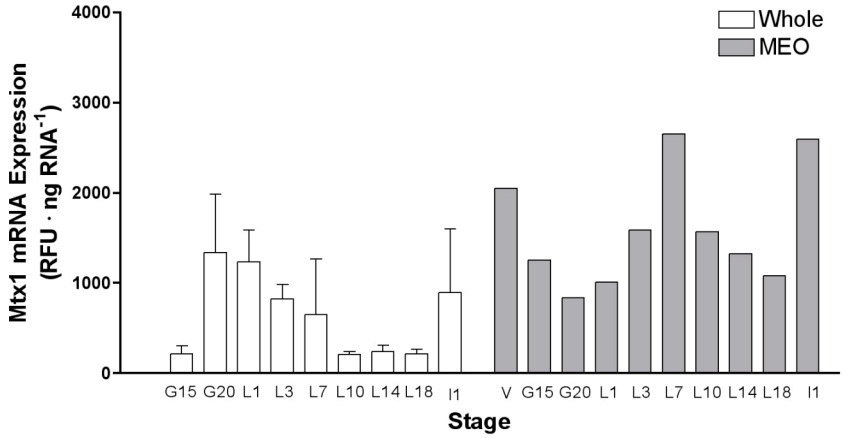


FIGURE 4.11 - mRNA expression analysis of the methotrexate carrier, Mtx1, in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue and isolated mammary epithelial organoids from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

4.1.3.8. The Divalent Metal Transporter (Dmt)

Dmt1 Transport Protein Expression in Whole Mammary Gland Tissue

The message for Dmt1 remains relatively constant from mid gestation period until day 3 of lactation, however, mid lactation period showed a decrease in expression to less than 50% of values seen during gestation and early lactation stages (L3), which remained constant thereafter (Figure 4.12).

Dmt1 Transport Protein Expression in Mammary Epithelial Organoids

The Dmt1 transporter in MEO shared certain similarities with whole tissue preparations in expression level and pattern during early lactation stages, however this similarity breaks down in later stages of lactation. Message levels of Dmt1 deviated very little from virgin tissues into day 15 lactation, however declined rapidly thereafter to ~55% virgin levels by day 20 of gestation. Dmt1 transcript levels remained steady, thereafter, into early lactation. However, levels declined rapidly during mid- and late stage lactation such that at day 10, 14, and 18 day lactation, transcript levels have decreased by ~53%, ~72%, and ~95% relative to day 1 of lactation. mRNA expression

recovers at the onset of involution with an increase to ~35% of the level seen in virgin tissues (Figure 4.12).

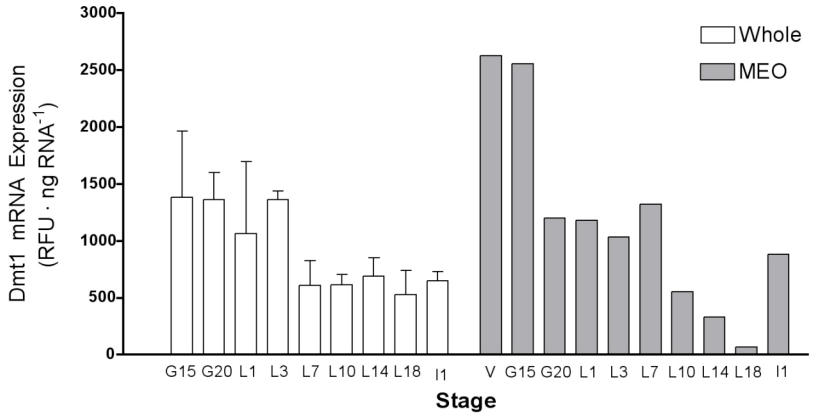


FIGURE 4.12 - mRNA expression analysis of the divalent metal transporter, Dmt1, in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue and isolated mammary epithelial organoids from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate).

4.1.4. mRNA Expression Kinetics of the Milk Protein β-Casein

β-Casein Protein Expression in Whole Mammary Gland Tissue

The message encoding the milk protein, β-casein was increased throughout gestation where levels at very late gestation are nearly double (~90% higher) the level seen at mid gestation (G15; Figure 4.13). β-casein mRNA increased from day 1 of lactation by ~65% at day 3 where it began to decline to reach values ~40%, and ~10% greater than day 1 at days 7 and 10 of lactation, respectively. Day 14 showed an even further decline in mRNA levels to reach ~75% of lactation day 1 values, which was followed by another fluctuation in mRNA that increases to reach ~125% of day 1 values. With involution β-casein mRNA decreased to levels ~77% of lactation day 1

values. The interindividual variation in β-casein expression, appear to be far greater than any of the transport proteins analyzed.

β-Casein Protein Expression in Mammary Epithelial Organoids

β-casein expression was far lower in MEO than whole mammary gland RNA and in addition followed a far different expression pattern at all time points analyzed. β-casein mRNA increased markedly with the onset of pregnancy with levels exceeding virgin tissue levels by more than 900% (~2800% and 928% greater than virgin at gestation day 15 and 20 respectively). β-casein mRNA level continued to rise into early lactation where levels exceeded virgin tissues by more than 3800% by day 3 of lactation. However, mRNA levels drop off immediately at mid-stage and late stage lactation reflecting similar levels as seen in the quiescent state. With involution a small recovery of mRNA was observed to reach levels ~160% greater than virgin tissues (Figure 4.13).

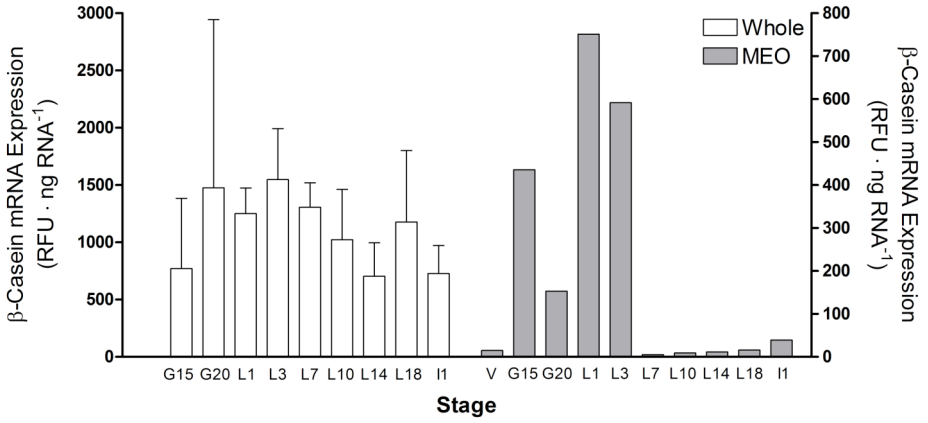


FIGURE 4.13 - mRNA expression analysis of the milk protein, β-casein in whole mammary tissue (Whole, open bars) and isolated mammary epithelial organoids (MEO, shaded bars). Total mRNA was isolated from whole mammary tissue (Whole) and isolated mammary epithelial organoids (MEO) from virgin female Sprague-Dawley rats and dams at various stages of gestation (G15, G20), lactation (L1, L3, L7, L10, L14, L18), and involution (I1). Whole tissue and MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) where whole tissue results are expressed as mean ± S.D. (n = 4/group) and MEO results are expressed as the mean of duplicate pooled MEO samples (n = 1; 4 individual samples, pooled and analyzed in duplicate). MEO expression data are represented by the right y-axis.

4.2. Primary Mammary Epithelial Organoid (MEO) Cell Culture System

4.2.1. Structural Architecture of Plated Mammary Epithelial Organoids (MEO)

Primary MEO plated using a Matrigel™ coated cell culture plate and a 2% Matrigel™ overlay developed into lobular (A), multilobular (B), or lobuloductal (C) structures resembling structures seen in the mammary gland during lactogenesis *in vivo* (Figure 4.14). Furthermore, cultured MEO from young differentiated MEO (L1) appeared to maintain structural architecture up to 4 days of culture. However, by day 8, there appears to be a marked reduction in viable structures (Figure 4.15).

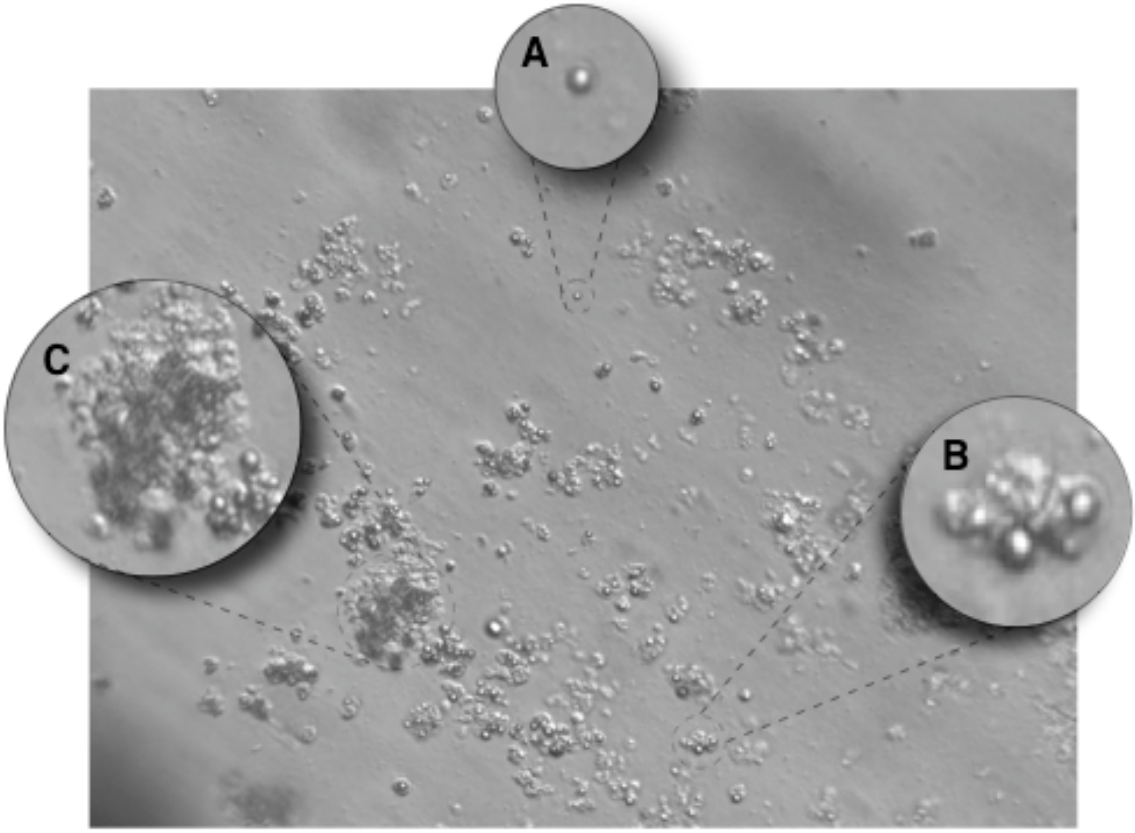


Figure 4.14 – Light microscopic appearance of isolated mammary epithelial organoids (MEO) plated on a thick Matrigel™ coated cell culture dish with a 2% Matrigel™ overlay. Plated MEO develop into lobular (A), multilobular (B) or lobuloductal (C) structures resembling active milk-producing structures seen during lactogenesis *in vivo*. MEO were isolated from a lactating female Sprague-Dawley rat at day 1 of lactation and shown at day 5 of culture. The background image represents a 10× magnification, whereas the larger insets are merely present to emphasize the developed MEO structures and do not represent any quantifiable magnification.

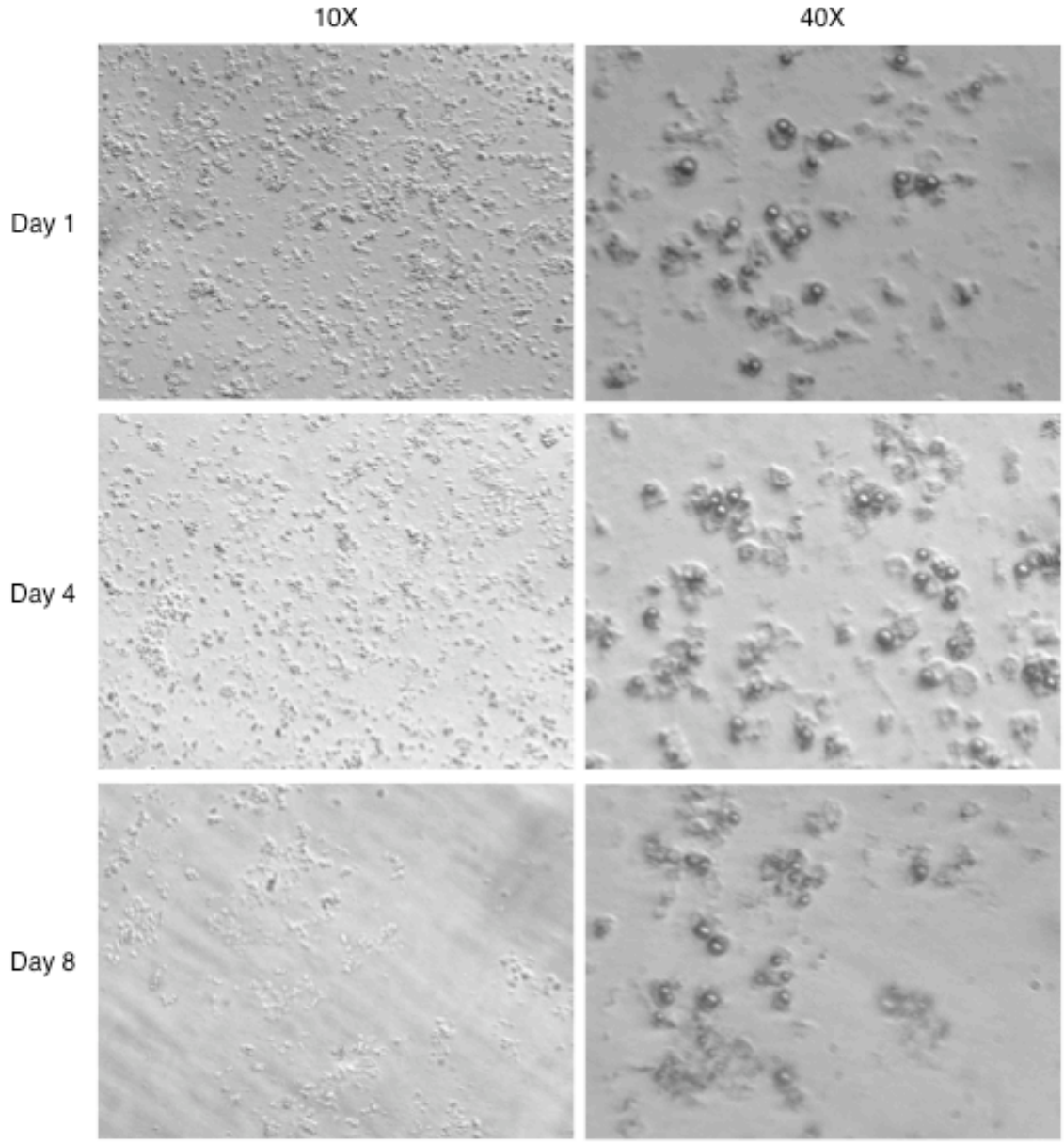


Figure 4.15 – Light microscopic appearance of isolated mammary epithelial organoids (MEO) after a plating duration of 0, 4, and 8 days on a thick Matrigel™ coated cell culture dish with a 2% Matrigel™ overlay. MEO were isolated from a lactating female Sprague-Dawley rat at day 1 of lactation and plated on a thick Matrigel™ coated cell culture dish with a 2% Matrigel™ overlay.

4.2.2. Transporter mRNA Expression in a Primary Mammary Epithelial Organoids (MEO) Cell Culture System

The mRNA expression of a select assortment of transporters (Mrp1, Ent1, Cnt3, Octn2, Octn3, Pept2) and the milk protein β-casein in MEO were assessed for their ability to maintain *in vivo* transporter expression after time in culture (4 or 8 days) relative to freshly isolated MEO.

Mrp1 mRNA expression changed markedly throughout the plating period for MEO plated at both lactation day 1 and lactation day 10. Relative to freshly isolated MEO from day 1 of lactation, 4 days of plating decreased Mrp1 levels by ~70%. This decrease continued until day 8, where harvested MEO expressed Mrp1 mRNA at levels less than 15% of those seen in fresh MEO. MEO isolated from lactation day 10 showed a similar trend of expression as those from day 1 of lactation. Relative to freshly isolated MEO from lactation day 10, 4 days of plating decreased mRNA expression levels by ~55%, and by 8 days in culture Mrp1 expression in day 10 MEO was less than 30% of the expression seen in fresh MEO (Figure 4.16A).

Cnt3 mRNA changed markedly for MEO plated from day 1 of lactation. Relative to freshly isolated MEO, 4 days in culture dropped Cnt3 mRNA levels by ~ 95%, and by 8 days was less than 1% of the levels seen in fresh MEO. MEO plated from day 10 of lactation showed a similar pattern of decline; however nowhere near the magnitude as early stage MEO. After 4 days in culture, Cnt3 mRNA levels decreased ~ 15% from freshly isolated MEO, and by 8 day of culture, mRNA levels declined further to reach 40% of the level seen in fresh MEO (Figure 4.16B).

Octn2 mRNA expression in cultured MEO from day 1 of lactation showed a marked increase following 4 days in culture to reach levels greater than 400% of those seen in fresh MEO. By day 8 of culture levels return to a level similar to those seen in fresh MEO (~ 38% greater than fresh MEO). Day 10 MEO in culture showed a similar yet less dramatic change in mRNA levels. Four days in culture increased Octn2 mRNA levels by ~22% followed by a decline to day 8 where levels were nearly equal to fresh MEO (~ 1.5% greater than fresh MEO; Figure 4.16C).

Octn3 mRNA levels in MEO from day 1 of lactation increased throughout the plating period. Day 4 of culture revealed an increase in Octn3 mRNA of ~ 82% which

continued to rise and by 8 days in culture the mRNA levels were beyond 600% of those seen in fresh MEO. Cultured MEO from day 10 of lactation decreased slightly by day 4 of culture (~18% decrease relative to fresh) and then recovered by culture day 8 to reach levels ~15% greater than fresh MEO (Figure 4.16D).

Ent1 mRNA expression in cultured MEO from day 1 of lactation increased dramatically after 4 days in culture to reach levels ~225% higher than fresh MEO. The expression level by day 8 of culture decreased markedly from day 4 of culture (~90%) to reach levels equivalent to ~40% of the level seen in fresh MEO. Cultured MEO from day 10 of lactation showed a similar pattern of expression throughout the plating period as MEO from day 1 of lactation, however at a much lower magnitude. Four days in culture increased mRNA expression by ~160% relative to fresh MEO, followed by a decline of ~72% (relative to day 4 of culture) to reach levels ~ 30% lower than fresh MEO (Figure 4.16E).

Pept2 mRNA expression in MEO from day 1 of lactation increased slightly following 4 days of cell culture (~27% relative to fresh MEO), however declined thereafter to dip below fresh MEO levels by ~40% after 8 days in culture. mRNA expression in cultured MEO from day 10 of lactation increased more than 380% from fresh MEO after 4 days in culture, which was followed by a decrease in expression at day 8 of culture (~65% of the expression seen in culture day 4) to reach levels exceeding fresh MEO by more than 220% (Figure 4.16F).

The message encoding the milk protein β-casein in both young (L1) and older (L10) differentiated MEO decreased more than 98% after 4 days in culture. Following 8 days of culture duration, the message in young MEO was decreased to less than 1% of freshly isolated MEO, and the message encoding β-casein in older MEO dropped to visible levels that lay below the quantification level of the assay (Figure 4.16G).

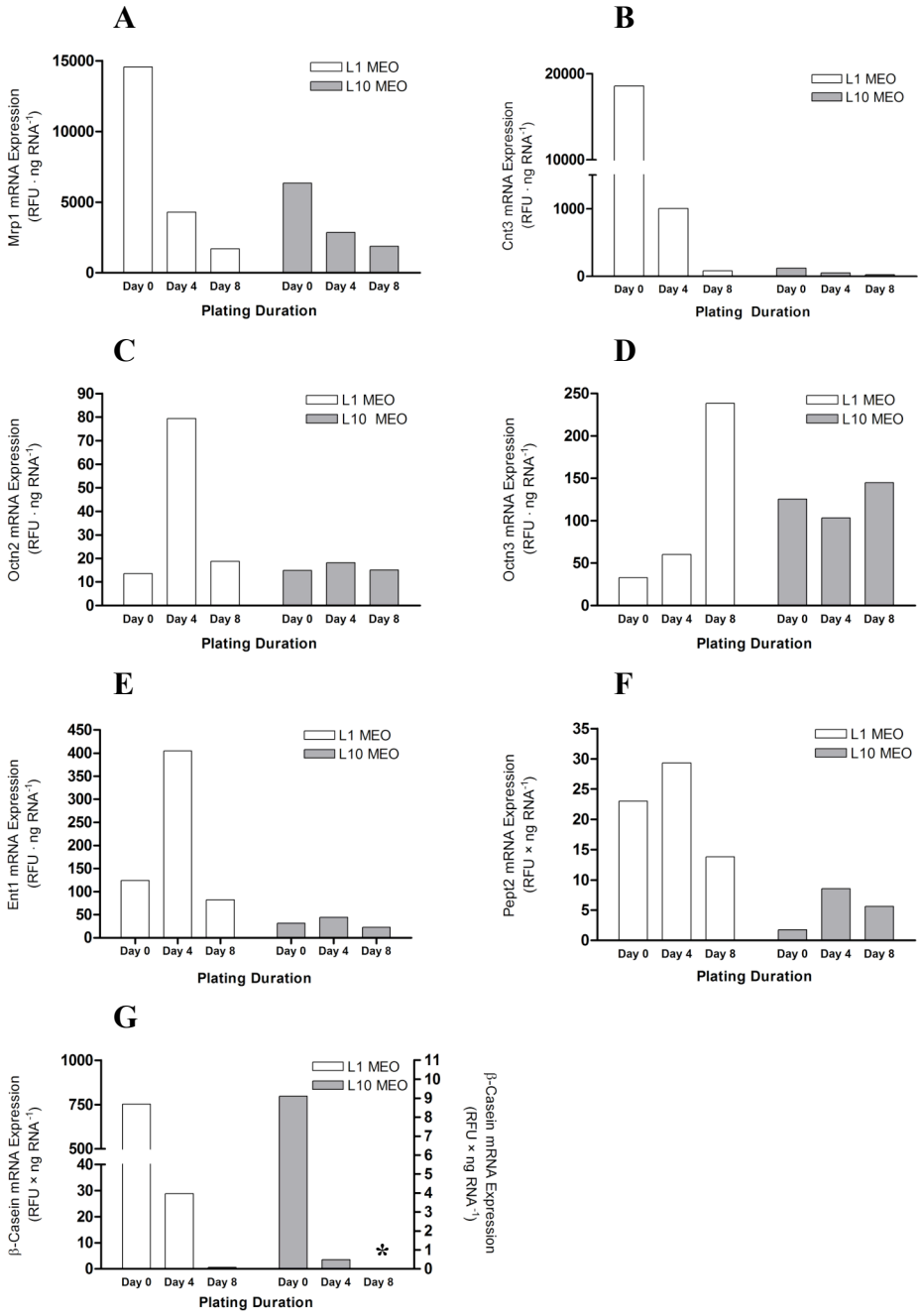
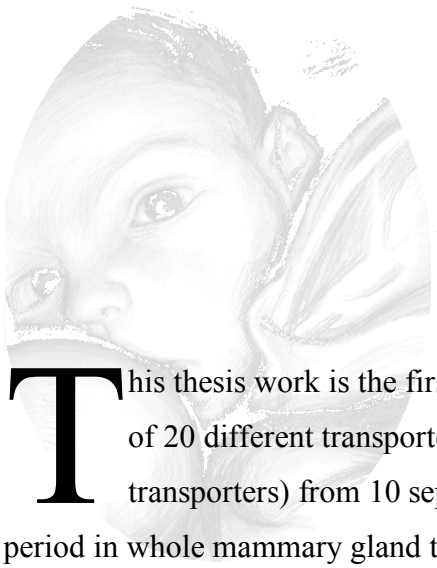


Figure 4.16 – mRNA expression of Mrp1 (A), Cnt3 (B), Octn2 (C), Octn3 (D), Ent1 (E), Pept2 (F), and β-casein (G) in cultured primary mammary epithelial organoids (MEO) after 4 or 8 days in culture. MEO were isolated from female Sprague-Dawley dams at day 1 (L1; empty bars) and day 10 (L10; shaded bars) of lactation and plated on 35mm Matrigel™ coated cell culture plates with a 2% Matrigel™ overlay. Total RNA was isolated after 4 days or 8 days in culture where MEO data is expressed as relative fluorescence units (RFU) normalized to total RNA (ng) as the mean of duplicate MEO samples (n = 1/group). * β-casein expression dropped below quantifiable levels by day 8 of culture in L10 organoids.



- V -

Discussion

This thesis work is the first to evaluate the mRNA temporal expression kinetics of 20 different transporters (3 xenobiotic transporters and 17 nutrient transporters) from 10 separate transporter families throughout the lactation period in whole mammary gland tissue and isolated populations of milk-secreting epithelial cells. Characterization of mRNA levels of nutrient transporter genes in the lactating mammary gland may provide insight into the major proteins involved in the accumulation of nutrients in the breast milk. More specifically, the expression patterns of transporter genes throughout lactation may identify the critical windows of nutrient delivery to the neonate during the suckling period. Knowledge of transporter gene expression throughout lactation is a necessary first step in identifying the pivotal role of active transport proteins in the transepithelial movement of nutrients across the lactating mammary epithelia. Furthermore, it allows the identification of when xenobiotic-nutrient transporter interactions may have the greatest impact on milk composition during lactation.

Major findings from this thesis demonstrate a complex pattern of gene expression in the mammary gland throughout lactation to provide adequate nutritional stimuli to the suckling infant. This gene expression differed markedly between whole tissue preparations and isolated milk-secreting epithelial cells for β-actin, Mdr1a, Mdr1b, Oct1, Octn3, Ent3, Cnt1, Cnt3, Ncbt1, Pept2, Mtx1, and β-casein. This brings

into question whether whole mammary gland tissue is truly appropriate for an understanding of transporter expression in the mammary epithelium. Since the MEO more accurately represents the physiological barrier between the maternal and neonatal system, the gene expression in MEO may better represent the transfer of nutrients across the blood-mammary gland barrier. Furthermore, MEO cultured in an extracellular matrix-rich environment maintained transporter expression at the mRNA level, emphasizing the potential of a primary MEO *in vitro* model system to screen possible xenobiotic-transporter interactions at the lactating mammary gland.

5.1. Transporter mRNA Expression Kinetics in Whole Mammary Tissue Versus Isolated Mammary Epithelial Organoids (MEO)

To determine the changes in transporter expression during different stages of mammary gland differentiation and lactation, I used real-time QRT-PCR analysis on whole mammary gland tissue and isolated mammary epithelial organoids (MEO). Both preparations were assessed since the literature only reports transporter expression from whole mammary gland preparations[31, 52, 71, 73]. Yet MEO preparations contain principally mammary epithelial cells, which are the relevant cell type when considering solute transport across the blood-mammary gland-barrier. Consistent with the hypothesis, the temporal mRNA expression patterns and levels of transporters from the ATP-binding cassette (ABC) and the solute carrier (SLC) family exhibit important differences between whole mammary gland tissue and isolated milk-producing cells (MEO). Similar temporal expression patterns and relative expression levels between whole tissue and MEO do exist for Mrp1, Octn1, Octn2, Ent1, Ent2, Cnt2, Ncbt2, and Dmt1 (Figures 4.4, 4.6A, 4.6B, 4.7A, 4.7B, 4.8B, 4.9B, & 4.12, respectively). However, the remaining transporters/proteins show either a similar temporal pattern with markedly differing relative expression levels (Mdr1a, Mdr1b; Figure 4.3), or markedly different temporal patterns and relative expression levels (β-actin, Oct1, Octn3, Ent3, Cnt1, Cnt3, Ncbt1, Pept1, Pept2, Mtx1, β-casein) (Figures 4.1, 4.5, 4.6C, 4.7C, 4.8A, 4.8C, 4.9A, 4.10, 4.11, & 4.13 respectively). Such discrepancies beg the question as to which of these two preparations, whole mammary gland or MEO, most accurately reflects the functional capacity of the mammary epithelium *in vivo*. Since the whole mammary

gland is composed of a heterogeneous population of cells (adipose cells, myoepithelial cells, immune cells, milk-producing epithelial cells, fibroblasts), it may not accurately represent the true nature of the transport processes within the mammary epithelium. Unfortunately, no studies to date discriminate between various mammary gland preparations. Interestingly, similar discrepancies have been evaluated for various transporter mRNA levels in the testis of male Sprague-Dawley rats. Isolated Sertoli cells showed a higher expression of transporter mRNA for Mdr2, Mrp1, Mrp4, Mrp6, Mrp7, Mrp8, Tst1, Tst2, Oatp2, Oatp12, Oct3, Octn2, Dmt1, Wilsons, Znt1, Ent1, and Ent2 than did a crude testis tissue extract[208]. On the other hand, Sertoli cells and whole testis extracts show a similar level of expression for numerous other transporters (Mdr1a, b; Mrp2, 3, 5; Bsep; Ntcp; Ibat; AbcG5, G8; Oatp1, 3, 4, 5, 9; Pgt,; Oat1, 2, 3, k; Oct1, 2; Octn1; Menkes; Cnt1, 2; & Pept1, 2). These results support the expression data of the current study where a tissue source composed of a heterogeneous population of cells types may exhibit important differences in transporter expression when compared to isolated cell populations, depending on the localization of these proteins (i.e. barrier epithelium or ubiquitously expressed). In the current study isolated MEO show a higher expression (β-actin, Mdr1a, Mdr1b, Octn3, and Cnt1), lower expression (Oct1, Ent3, Cnt3, Ncbt1, Pept2, and β-casein), or similar expression (Mrp1, Octn1, Octn2, Ent1, Ent2, Cnt2, Ncbt2, Pept1, Mtx1, and Dmt1) of transporters relative to whole tissue extracts.

Although no further studies were conducted to assess whether MEO or whole mammary gland best reflected the transport function of the mammary epithelium, such discrepancies may give insight into the tissue localization of these transporters. Expression levels that are higher or in certain instances similar between MEO and whole tissue may be indicative of transporters that are exclusively localized to the milk-producing epithelia. For instance, the iron transporter, Dmt1, is exclusively localized to the polarized epithelia of the mammary gland[52] and shows similar expression levels between isolated MEO and whole tissue. Therefore, whole tissue RNA data may be appropriate for evaluating proteins expressed in the major cell type of a tissue (i.e. MEO in the mammary gland). On the other hand these results may be misleading for ubiquitously expressed transporters where the individual cell types of the mammary

gland each expresses their own compliment of transporters. For instance, isolated adipocytes (a major component of the mammary gland) have active transport processes for glucose[209], insulin and IGF-1[210], vitamin C oxidation products[211], vitamin D3 and E[212], and calcium[213]. Similarly, myoepithelial cells, which contribute to the milk-ejection reflex, have transport mechanisms for manganese, calcium, and potassium[214, 215].

Although several transporters may be localized to these secondary cell types in the mammary gland, the expression of transporters in isolated MEO underlies the primary site of nutrient transport into the mammary gland lumen. Therefore, evaluating a whole tissue extract in an investigation of processes within the milk-producing cells of the mammary gland may either dilute the target RNA or contribute to the specific transporter RNA pool and yield erroneous, unreliable results. However, we lack necessary scientific investigations into the localization of these transporters within the mammary gland. Therefore, without immunohistochemical determination, or analyses of molecular markers that are specific to certain cell types (i.e. cytokeratins), such conclusions remain speculatory. The remainder of this thesis work will focus on the transporter expression kinetics in mammary epithelial organoids (MEO), giving reference to the whole mammary gland only where applicable, since we are most concerned with the transport processes in the mammary epithelial cells and not the whole mammary gland.

5.2. Temporal Expression Patterns of ABC-Transporters Throughout Lactation in Mammary Epithelial Organoids

Transporters of the ATP-binding cassette superfamily (Mdr1a, Mdr1b, & Mrp1) follow a distinct decrease in expression throughout lactation. Since these transporters serve to actively remove large lipophilic molecules and xenobiotics from the cell, this finding may provide insight into the protective mechanisms adapted at the mammary gland to safeguard neonatal health. The decline in expression throughout lactation may not be protective for the mammary epithelia due to a decreased expression of the basolateral efflux proteins (i.e. Mrp1, which is expressed basolaterally in most other barrier epithelia), however is protective to the nursing neonate by limiting drug exit

through the apically expressed proteins (i.e. Mdr1a & Mdr1b) at the polarized mammary epithelium. Furthermore, rat mammary gland tissue has its own compliment of drug metabolizing enzymes (cytochrome P450; CYP) and during pregnancy total CYP content increases significantly in the breast tissue and continues to rise and peak during lactation[216, 217]. The increase in total CYP content includes the increase of many members of the CYP family, including CYP1A1, 2E1, 2D1, 2D3, 2D4, and 4A3. Conversely, there is a decrease in expression of many other CYP enzymes (CYP2A, 2B, and 3A). However, these enzymes are present at low levels have been shown to undergo transcriptional upregulation if challenged with a xenobiotic insult[216]. Despite a possible decrease in basolateral efflux transporter expression during lactation, the mammary epithelium has mechanisms to deal with xenobiotic insults that might arise. Such changes in xenobiotic metabolizing enzymes and transporter expression suggest the mammary gland undergoes substantial regulation.

Little is known about the individual mechanisms for regulation of gene expression in the mammary gland. It is often avoided by mentioning the possibility of the mammary gland undergoing a complex process of endocrine regulation and since the mammary gland is a hormonally-driven system, it is clear that endocrine signals must play a part in regulation[22]. However, with little information available on the effects of individual hormones on mammary gland processes (specifically protein-mediated transport) one cannot draw many direct correlations. Substantial evidence has highlighted the importance of extracellular matrix (ECM) interactions in the regulation of gene expression in the mammary epithelial cell[218-222]. The mammary gland ECM composition changes markedly with reproductive state[223], presumably due to certain hormonal patterns. ECM components are known to be responsible for the regulation of intracellular P-glycoprotein mRNA (specifically laminin and collagen IV components of ECM)[224], possibly due to cell-ECM interactions through cell surface receptors (i.e. integrins) which exploit a number of signal transduction pathways[222, 225]. Therefore, as the mammary gland ECM environment matures, it may regulate the expression of many transporters through its interactions with cell surface receptors.

Taken together, these data demonstrate that the mammary gland reduces the expression of these important players in xenobiotic transport and disposition to limit

drug exposures to the neonate. In addition, the mammary gland also upregulates its own compliment of drug metabolizing enzymes, some of which undergo transcriptional regulation in the face of a xenobiotic attack, which allows for the metabolism of xenobiotics that may enter the mammary epithelia. The reduction in the mRNA levels of the ABC transporters is consistent with the primary role of the differentiated mammary epithelium to provide a nutrition source that preserves the health of the neonate (i.e. nutrition, immunology, bioactive agents, etc.) and relatively low levels of potentially harmful xenobiotics. The decline in the expression of the ABC transporters indicates that all the resources of the epithelial cells are dedicated to providing nutrient-rich and safe secretions. This is strong evidence that the mammary gland has strictly evolved mechanisms to produce and provide an unparalleled nutrient source that meets the health needs of the developing offspring and therefore, breast milk is under most circumstances the safest form of infant nutrition.

5.3. Temporal Expression Patterns of SLC-Transporters Throughout Lactation in Mammary Epithelial Organoids

5.3.1. The Organic Cation Transporter Family (Oct)

The first member of the organic cation transporter family (Oct1) was the only member of this family to undergo significant changes between non-lactating and lactating mammary epithelial cells[3] and therefore, was the only member of this family to be investigated.

Oct1 is a facilitated organic cation transporter[88] that is expressed at the basolateral membrane of both the hepatocyte and the proximal tubule in the kidney[88, 226]. The expression of Oct1 in MEO is much lower than that observed in whole tissue giving an indication that Oct1 is expressed ubiquitously in the mammary gland as it's expression in found in a wide range of tissue types[202]. Oct1 expression in whole tissue indicates its critical endogenous role in the mammary gland in the later stages of lactation, possibly due to the mobilization/utilization of fat. Oct1 has been shown recently to be capable of transporting prostaglandins[227] and therefore, may be capable of transporting other positively charged fatty acid components involved in lipolysis in the mammary gland, however this claim has yet to be substantiated. Oct1 in MEO

demonstrates that the facilitated transport of cations is critical in early lactation stages (L1 – L7) and of less importance in later stages of lactation (Figure 4.5). Recently, the breast milk content of numerous micronutrients has been evaluated between colostrum and mature milk[228], and the increase in some of these charged moieties may be due, at least in part, to the contributions of organic cation transporters, particularly following the establishment of tight junctions and the formation of a polarized mammary epithelium.

5.3.2. The Organic Cation/Carnitine Transporter Family (Octn)

The Octn family of transporters are responsible for the uptake of numerous charged cations, and are one of the primary transporter families which facilitate the uptake of dietary carnitine, a conditionally essential nutrient[88, 89]. More importantly, the newborn is not capable of carnitine synthesis during the initial months of life[229, 230], and must therefore rely solely on exogenous sources of carnitine during this period. Consequently, the transporters responsible for the accumulation of carnitine in breast milk are of critical importance.

Octn1 is the only member of the Octn family that uses a bi-directional transport mechanism that is independent of sodium[88], whereas Octn2 and Octn3 are the proposed dominant players for carnitine transport which employ a sodium and pH-dependent mechanism[88, 89]. Since Octn1 has a lower affinity to transport carnitine, and is a bi-directional transporter, it may only play a very minor part in the flux of carnitine into breastmilk[88]. Accordingly, MEO data shows a relatively constant expression level into late lactation, which seems reasonable for a facilitated transporter (Figure 4.6a). Knowledge of the localization of Octn1 at the apical or basolateral membrane of the polarized mammary epithelium will further clarify its role during lactation.

Octn2 and Octn3 are the high[88] and intermediate-affinity[89] carnitine transporters respectively, and are therefore the most likely transporters responsible for the milk levels of carnitine. Milk carnitine is constant for the first 3-weeks postpartum in humans where levels then significantly drop (almost in half) to remain lower and constant for the remainder of lactation[231]. Similarly, mammary gland explants from lactating rats showed a significant drop in L-carnitine uptake from early lactation to late lactation[232]. The mRNA expression pattern of Octn2 reflects this decline in both whole

mammary gland tissue and MEO and may therefore be the main contributor to milk carnitine status. The expression pattern of Octn3 differs markedly as Octn3 increases steadily throughout lactation to greatly exceed Octn2 levels. Since Octn3 is a relatively new transporter, there is substantially less literature available, however it has recently been reported that Octn3 is a transporter localized to the peroxisome[233] of mammalian cells. Since the peroxisome is a metabolic organelle, Octn3 may only play a critical role in transporting the chain-shortened products of lipid metabolism from the peroxisome back into the mammary gland cells[233] and therefore may only contribute to cellular energy status and may not be a major contributor to the carnitine content of breast milk.

The tissue distribution of Octn3, the intermediate carnitine transporter[233], is limited and has been shown to be almost exclusively expressed in the testis of mice[234], the basolateral compartment of chicken enterocytes[89], and localized to the peroxisome in human skin fibroblasts[233]. Since its expression is very limited, it provides insight into its significant role in the mammary gland. However the identification of where Octn2 and Octn3 are expressed in the polarized epithelium will help to clarify their roles during lactation.

5.3.3. The Concentrative Transport Family (Cnt)

The concentrative nucleoside transporters all share the same sodium-dependent, active transport mechanism. However, the substrate profile for each transporter is slightly different. Cnt1 transports the pyrimidine nucleosides and adenosine[85], Cnt2 transports the purine nucleosides, uridine[85], and most recently has been shown to be a high-affinity adenosine transporter[90], and lastly, Cnt3 transports both purine and pyrimidine nucleosides[85]. In addition, the cellular localization of mammalian nucleoside transporters has been extensively reviewed and the exclusive expression of these transporters to the apical membrane of absorptive epithelia indicates their role in the apical uptake of nucleosides and nucleoside analogues[235, 236]. However, this uptake role is not limited to absorptive epithelia (i.e. intestine). Secretory epithelia, such as the renal and hepatic barriers, are responsible for the cellular removal of toxins. Yet these barriers are still capable of lumen-facing cellular uptake of nucleosides and therefore, the cellular distribution and function of the nucleoside transporters is identical to that in the absorptive epithelia[237, 238]. The universal cellular expression and function of

nucleoside transporters implies their role in the cellular accumulation of nucleosides, and the diversity in the substrate profile indicates a distinct role for each individual transporter.

Cnt1 mRNA increases steadily to mid-lactation where it remains relatively constant until involution in MEO. This expression appears to be primarily in the MEO as whole tissue levels of Cnt1 are nearly 100-fold lower (Figure 4.8A). Since total nucleosides/nucleotides in milk tend to decrease over the course of lactation[239], Cnt1 must be involved with other endogenous roles within the mammary gland, possibly involving the low-affinity transport of adenosine or adenosine analogues such as cyclic-AMP (cAMP). Adenosine levels in human milk are relatively low (~16% of total potentially available nucleotides[240, 241]), however since Cnt1 is one of the only nucleoside transporter capable of the active transport of adenosine, it must play a role in determining adenosine concentrations in milk. The adenosine analogue, cAMP, shows an increased binding affinity with pregnancy, which is further increased in lactation with highest levels in early involution[242]. The increase in cAMP binding during pregnancy and involution is strongly associated with the level of cell proliferation and this is possibly representative of Cnt1 mRNA expression in MEO. In addition, elevated levels of cAMP and accumulation in extracellular fluids in mouse mammary glands decreases the responsiveness of the epithelial cells to lactogenic hormones and turns off β-casein gene expression[243], which may explain the marked increase in Cnt1 mRNA levels during involution. Furthermore, cAMP has been shown to increase the ability of the small intestine to transport fructose without increasing GLUT mRNA levels[244] and therefore, Cnt1 may play a role in modulating sugar uptake in the neonatal small intestine. The possible role of Cnt1 in mediating cAMP transport has not been evaluated, however the ability of this transporter to accommodate numerous nucleoside analogues[161] does indicate its diversity and possible capability of mediating cAMP transport.

The remaining nucleoside transporters, Cnt2 and Cnt3 follow a similar pattern of expression representative of the decline in total nucleosides in milk fractions throughout lactation[239]. The dramatic upregulation of Cnt2 and Cnt3 during early lactation are likely indicative of the critical role nucleosides play in the nursing infant. Feeding a

nucleoside and nucleotide mixture in addition to normal diet to male rats caused a significant increase in jejunal weight, protein and DNA content, villous height, and a decrease in tight junction size between enterocytes[245]. Furthermore, dietary nucleosides caused an increase in B- and T-helper cell antigens in mice and contributed to the maturation of intestinal lymphocytes and ultimately intestinal immunity[246]. In addition, Cnt2 has been shown to transport cyclic ADP-ribose (cADP-R), a calcium mobilizing second messenger[247] which has been shown to modulate intracellular calcium status by activating calcium release through intracellular ryanodine receptors[247]. Therefore Cnt2 may also play a role in myoepithelial cell contraction by increasing the frequency of calcium sparks in the sarcoplasmic reticulum, leading to an increased frequency of myoepithelial cell contraction[248] and a consequent increase in milk-ejection. The ability of Cnt2 to transport adenosine was recently evaluated, and was shown to be the highest affinity transporter for the adenosine substrate[90]. The role of adenosine in maintaining mammary gland functions is unknown. However, adenosine has been shown to be a regulator of cellular proliferation in cardiac fibroblasts[249] and vascular smooth muscle cells[250]. It may be possible that intracellular accumulation of adenosine into mammary epithelial cells, via Cnt2, may decrease extracellular concentrations (where it accumulates during normal metabolic activity[251]), thereby promoting cell proliferation in the surrounding tissues (parallel to the correlation of Ent1 and cellular proliferation). The decrease thereafter, may allow for the extracellular accumulation of adenosine, which inhibits cellular proliferation[249, 250]. On the other hand, Cnt2 may only be involved in the transepithelial flux of nucleosides into the breast milk and therefore, a major regulator of the adenosine composition in breast milk.

The limited tissue distribution of these transporters indicates the significance of their expression in the mammary gland, especially Cnt3, which is expressed at very low levels and in very few tissues compared to Cnt1 and Cnt2[204]. Therefore these transporters must play a critical role in the mammary gland by providing critical nucleosides to the neonate, or regulating mammary epithelial cell status, or both. Recent research demonstrates that a differentiation stimulus (glucocorticoids) presented to rat intestinal epithelial cells sparked a significant increase in Cnt transporter function at all 3 functional levels, mRNA, protein, and activity for both Cnt1 and Cnt2[252].

Therefore, the distinct patterns of Cnt expression, specifically Cnt1 and Cnt2, which may represent an increasing differentiation stimulus and an inhibiting proliferation stimulus (via adenosine) respectively, indicates a complex pattern of regulation which is not understood in the mammary gland. Nevertheless, the capacity of these transporters to facilitate the transepithelial movement of nucleosides to assist in breast milk composition cannot be ignored. Dietary nucleosides are involved in a number of key biochemical processes such as energy metabolism (ATP & GTP), coenzyme systems (FAD, NAD & NADPH) and second messenger systems (cAMP & cADP-R) and are considered conditionally essential to the nursing neonate due to the large energy demands of *de novo* synthesis[253].

5.3.4. The Equilibrative Nucleoside Transport Family (Ent)

The equilibrative nucleoside transporters are proposed to work in tandem, as bi-directional facilitators, with the Cnt's to maintain nucleoside flux across the cell barrier[86]. The tissue distribution of the Ent's are similar where they are all ubiquitously expressed and they share similar substrate profiles, with the exception of Ent1 which transports purine and pyrimidine nucleosides but does not transport nucleobases[86].

The temporal expression patterns of the Ent's in the mammary gland are similar with the exception of Ent1, where Ent2 and Ent3 remain low in MEO and Ent1 is increased in early lactation. Since Ent1 does not allow the transport of nucleobases, the increase in early lactation may indicate that the smaller purine and pyrimidine nucleosides are of greater importance during early lactation and the other transporters capable of nucleobase transport are kept low and relatively constant. The altered expression of Ent1 may also reflect its coupled function to the Cnt transporters, Cnt2 and Cnt3, which show a similar expression pattern. However, such a relationship is purely speculatory. A more plausible explanation is the increase in Ent1 mRNA reflecting the proliferating nature of the mammary gland in early lactation. Isolated rat intestinal epithelial cells in the presence of a proliferative stimulus (EGF & TGF-α) showed a significant increase in Ent1 at mRNA, protein, and activity levels, however no alterations were visible for Ent2, at any functional level[252]. Therefore, the pattern of Ent1 mRNA may be representative of the proliferative status of the mammary gland where the rapid proliferation may provide the mammary gland with a "stockpile" of

undifferentiated cells. This "stockpile" can be accessed when older cells, near the end of their secretory lifespan, are sloughed off into the breast milk[254], at which time the differentiation status of the mammary gland would increase (as evident from Cnt1 data)[252].

Interestingly, Ent1 and Ent2 expression levels and patterns are similar between whole tissue and MEO, whereas Ent3 is expressed in a much different manner. The relative differences between whole tissue and MEO may give insight into the tissue specific expression of the Ent's within the mammary gland. Since the Ent's are ubiquitously expressed, a subtle difference in whole tissue levels (marginally higher) may indicate that Ent1 and Ent2 are also expressed in a secondary tissue that does not comprise a large percentage of the mammary gland. In the case of Ent3, it has recently been shown that Ent3 is localized to the intracellular membranes associated with the lysosome rather than the cell membrane as was originally proposed[255]. If the increase in energy demands during lactation causes fat mobilization within the mammary gland tissue, the increase of Ent3 mRNA in whole tissue may not be surprising as lysosomes are involved in a number of metabolic pathways, including lipolysis[256]. However, there is comparatively much less known about Ent3 than Ent1 and Ent2, and future studies are needed[86, 255].

5.3.5. The Nucleobase Transporter Family (Ncbt)

The nucleobase transporter family was originally called as such due to an amino acid similarity with a mammalian nucleobase transporter[257]. However, recent research suggests that the appropriate nomenclature remain as the sodium-dependent ascorbate transporters (SVCT) due to their inability to transport nucleobases[258]. Nevertheless, GeneBank database searches for rat nucleobase transporters result in cDNA sequences of the Slc23a family, which also encodes the mammalian sodium-dependent ascorbate transporters. With current research not supporting the Slc23a family's ability to transport nucleobases[258], it will be assumed that the nucleobase transporters are primarily ascorbate transporters and other mechanisms are involved in the transport of nucleobases in the mammary gland, similar to the mechanisms seen at the well characterized blood-testis barrier[259].

The levels of vitamin C in transition milk (colostrum) and mature milk are similar[260] and only begin to decline during prolonged lactation[261]. Therefore, transport mechanisms responsible for vitamin C should remain relatively constant for the majority of lactation and only decreasing into later stages, which is supported by the mRNA expression of the SVCT transporters in MEO. Interestingly, the expression pattern in whole mammary gland tissue is similar for Ncbt2, however not for Ncbt1, which shows a dramatic increase into the late stages of lactation, indicating an important endogenous role in the mammary gland. It was reported over two decades ago that the addition of ascorbic acid to rat smooth muscle cell culture systems altered the ECM matrix composition by increasing the ratio of collagen to elastin through reductions in elastin[261]. Similarly, cultured calf aortic smooth muscle cells demonstrated a similar increase in ECM collagen to be the dominant matrix component (greater than 80%) with ascorbic acid feeding[262]. In addition to modulating ECM composition in the calf smooth muscle cells, ascorbic acid feeding promoted the presence of poly(A+) RNA in the total RNA pool and therefore can modulate transcriptional changes in gene expression[263]. Both SVCT1 and SVCT2 demonstrate high affinity for L-ascorbic acid, with the latter transporter having a slightly higher affinity[91]. The endogenous role that each of these transporters play in the mammary gland is unknown, however, it may be possible that Ncbt1 regulates L-ascorbic acid transport to alter ECM composition in preparation for involution by decreasing cell responsiveness to hormonal signalling[264]. Further investigation is sorely needed.

5.3.6. The Oligopeptide Transporter Family (Pept)

The Pept transporters are a family of apically expressed transporters which are responsible for the uptake of small peptides from the lumen back into the cell for use in protein syntheis[92]. The presence and localization of Pept2, the high-affinity, low capacity peptide transporter has been previously investigated in the mammary gland and both Pept2 mRNA and protein was localized exclusively to the secretory cells. Pept2 is highly expressed at the apical cell compartment in numerous other tissues (i.e. kidney proximal tubule and choroids plexus[265]) and functions in the apical uptake of small peptides against an electrochemical gradient[266]. The function of Pept2 in other tissues implies its role in the apical uptake of short-chain peptides in the mammary gland

possibly resulting from the hydrolysis of milk proteins for use within the epithelial cells[73]. However, its role and expression throughout lactation is not understood. It may be possible that Pept2 is upregulated in early lactation to limit the presence of specific peptides in the milk until the gastrointestinal tract of the neonate adequately matures. Once the neonate intestinal system is ready, Pept2 may be limited to providing these specific small peptides to the epithelial cells of the mammary gland as the protein concentration of milk begins to increase throughout lactation[267]. Alternatively, Pept2 may merely provide the secretory mammary epithelial cells with specific proteins to maintain proper cell function[73].

On the other hand, the low-affinity, high capacity peptide transporter, Pept1, shows low expression throughout lactation with mRNA levels spiking during involution. Pept1 is also expressed at the apical cell compartment in the renal proximal tubules[268] and enterocytes[269] and is involved in the apical uptake of short chain peptides in a similar manner to Pept2. Pept1 may also be responsible for the uptake of a wide range of milk proteins from the mammary gland lumen, as it works in concert with Pept2. The spike in Pept1 mRNA in involution may imply its role in the less-selective uptake of milk proteins from the lumen in preparation for involution. However, these claims are speculatory and require further investigation.

5.3.7. The Methotrexate Carrier (Mtx1)

The methotrexate carrier (Mtx1) is the rat ortholog of the human reduced folate carrier and is responsible for the uptake of folate and anti-folates (i.e. methotrexate) in a sodium-dependent manner[93]. Folate is responsible for the maintenance of new cells, and is therefore critical in developmental stages where rapid cell division occurs (i.e. early life) due to its role in facilitating the replication of DNA[270]. The folate concentration in human milk varies greatly, however in all cases milk levels of folate increase throughout lactation, increase throughout the day, and increase in the transition from foremilk to hindmilk[271, 272]. The temporal expression of Mtx1 mRNA shows a continual increase until mid lactation where it begins to decline. It is not unreasonable to assume that the protein levels of Mtx1 remain elevated even after the gradual removal of the protein message (mRNA).

Conversely, the message encoding the methotrexate carrier in MEO may indicate its role within the proliferating mammary gland. Since folate is involved in cell proliferation, the increase in Mtx1 may indicate the needs of the mammary gland to support active cell proliferation during lactation. Throughout lactation, the mammary gland evacuates mammary epithelial cells that are near the end of their secretory lifespan and lays down new cell layers to maintain this rotation of cells[254]. Even though the total cell content of milk declines over lactation, and despite large interindividual variations[273], the percentage of total epithelial cell content tends to increase into mid lactation in humans[273]. The removal of older secretory cells to accommodate the newly proliferated cells may substantiate an endogenous role of folate to aid in the proliferation of the new secretory cells, similar to the possible role that Ent1 plays in active cell proliferation in early lactation. The decline into late lactation may indicate that there is a decrease in the proliferation of secretory cells and consequently may be a preparatory phase for the impending involution.

5.3.8. The Divalent Metal Transporter (Dmt1)

Dmt1 serves to actively transport non-heme (ferrous) iron into the breastmilk[52, 53] and is among the very few transporters which have been critically evaluated at the lactating mammary epithelia. The milk iron concentrations[54, 274], Dmt1 mRNA[50-52], and Dmt1 protein levels[50-52] all decline throughout lactation, and milk iron concentrations are highly regulated to be nearly independent of maternal iron status[52]. The decline in Dmt1 mRNA in previously published reports[52] was evaluated using a whole mammary gland RNA sample and is reflected in our results using whole tissue MEO. However, our whole tissue mRNA expression only marginally matches the previously published results. Although this is curious, an explanation may be hidden in the methodology. Previously published reports indicate that a subcutaneous dose of oxytocin was administered to stimulate milk let-down and a fraction of milk (~1mL) was taken from each animal prior to mammary gland excision[52] which may have limited luminal milk content. Our methodology did not involve milk removal. Since milk is composed of numerous, viable epithelial cells[254, 273], our whole tissue samples would have contained traces of milk[52] and blood fluids[275] that may have contributed to the Dmt1 signal, limiting the degree of similarity between previously published reports. Conversely, our

MEO samples were void of any milk or blood fluids and match previously published data. Since our whole tissue data was obtained using only a small section of mammary gland that may have had traces of milk and/or blood fluids, it is not surprising that our data does not match literature reports.

Using immunohistochemistry, Dmt1 was shown to be localized to the milk secretory cells and not present in the myoepithelial cells in the mammary gland[52]. Similarities with our MEO data may suggest that a whole tissue RNA sample might be appropriate in evaluating transporters that are localized to the milk-producing cells of the mammary gland. However, few other transporters have been evaluated in the mammary gland and therefore, each transporter would require a full characterization to substantiate this relationship.

5.3.9. The Milk Protein β-Casein

β-casein is a milk-specific protein that is expressed in the mammary gland during lactogenesis phase I and II[22] where mRNA levels increase significantly from virgin levels and remain elevated throughout the first 10-14 days of lactation where after it then begins to decline[276]. β-casein expression is dependent on ECM-cell adhesion interactions through the β-integrin signaling pathway[277] (more specifically, the interaction between these β-integrins and the extracellular component, laminin) and cell-cell contact[277]. Our whole tissue mRNA analysis shows a similar pattern of expression as seen in previous studies involving mRNA analysis[276], however our MEO data is far from equivalent. Possibly the procedure employed to isolate MEO from whole mammary gland tissue is sufficiently long and rigorous to nearly abolish milk protein expression by removing ECM-cell and cell-cell contacts. The critical interactions between ECM components and cell-surface receptors are processed by the cell within a few short hours[277]. The processing time for the isolation of MEO from whole mammary gland tissue is approximately 9 hours, during which, the dissociation of ECM-cell and cell-cell contact may turn off milk protein expression. Therefore, it appears that a whole tissue RNA analysis may be the most reliable way to evaluate milk-protein expression as organ architecture is preserved until RNA analysis.

Furthermore, a fresh, whole tissue preparation does contain luminal milk which is in part composed of viable mammary epithelial cells[254]. In some species (i.e. human)

these cells comprise over 90% of total milk cell counts[273]. These viable cells (nearly 100% viable in humans[273]) have been used in similar transporter evaluations using human milk[3], and have been used as a primary mammary epithelial source for cell culture and the evaluation of milk proteins such as the casein proteins[278]. Since these sloughed mammary epithelial cells are only present in a whole mammary gland preparation, we cannot ignore their contribution to the total RNA pool. Therefore, the results may not be representative of the β-casein protein expression in the polarized mammary epithelial cells of the mammary gland.

5.4. Temporal Expression Patterns In MEO: A Far Superior Tissue Source?

The temporal mRNA expression levels of transporters from the ATP-binding cassette family (ABC) and the solute carrier family (SLC) exhibit some differences between whole mammary gland tissue and isolated milk-producing cells (MEO) for the majority of transporters analyzed. These differences bring into question to what extent, if any, the isolation procedure might alter gene expression and to what extent the heterogeneous population of cells in the whole mammary gland influences expression patterns and levels of transporters in the mammary epithelium per se. It appears that the isolation procedure did not significantly alter transporter expression as evidenced from previously evaluated transporters at the lactating rat mammary gland[52]. Furthermore, the ability of a rigorous isolation procedure to alter gene mRNA expression was recently evaluated using whole liver tissue samples and isolated hepatocytes[279]. This rigorous isolation involving enzymatic digestion, did not affect gene mRNA expression[279]. One exception to this conclusion may be utility of using isolated MEO in the evaluation of milk-protein expression throughout lactation. Since milk-protein synthesis is rapidly regulated through cellular interactions with neighboring cells and extracellular matrix components (i.e. laminin)[277, 280], the removal of these interactions may rapidly alter cellular signaling. Consequently, MEO preparations may not allow for reasonable assessments of milk proteins.

With the possible exception of the milk protein, β-casein, our results, in addition to the remarkable similarities from previously published mammary gland reports[52] have lead us to believe that isolated MEO from whole mammary gland tissue may be a far superior tissue source for the evaluation of nutrient transporters during lactation.

Intuitively, of course, the isolated MEO seem to be a far superior choice as they are void of secondary mammary gland cell types and composed primarily of milk-producing epithelial cells, the key cell type in the evaluation of solute transporter across the mammary epithelia. However, MEO require much further characterization to substantiate this belief. Nevertheless, assuming our hypothesis is correct, the expression patterns of nutrient transporters from the ABC and SLC transporter superfamily in isolated MEO from whole mammary gland tissue follow 1 of 4 distinct temporal expression patterns throughout lactation: steadily decreasing throughout lactation (Mdr1a, Mdr1b, Mrp1 & Dmt1), increasing in early lactation and decreasing thereafter (Oct1, Octn2, Ent1, Cnt2, Cnt3, Pept2 & Mtx1), steadily increasing throughout lactation (Octn3 & Cnt1), and a relatively constant expression throughout lactation (Octn1, Ent2, Ent3, Ncbt1, Ncbt2 & Pept1). The distinct expression pattern may give insight into the temporal role of these transporters in establishing the maternal milk composition.

5.4.1. Decreased Temporal Expression Throughout Lactation

The only member of the SLC-transporter superfamily that undergoes a decline in expression in MEO throughout lactation is the divalent metal transporter, Dmt1. Dmt1 has previously been investigated in the mammary gland and the decline in Dmt1 mRNA is accompanied by a similar decline in Dmt1 protein[50-52] and in iron milk levels[54, 274]. The decline in all 3 elements of mammary gland iron begs the question of how the neonate responds to the decline of milk iron. The neonate is a very plastic system and undergoes many developmental changes in response to a number of stimuli, including nutritional stimuli. The decline in mammary gland iron status is accompanied by a gradual increase in intestinal iron transporters in the neonate in both rats[50, 51] and mice[281]. Therefore, the nutritional composition of the maternal milk may in fact be a signaling mechanism to the neonate to develop its own compliment of nutritional transporters. When iron status is high, neonate iron transporters are low, but the progression to a low iron status in the milk forces an upregulation of neonate iron transporters, a factor affecting the proper ontogeny of the neonatal system. Consequently, the expression pattern of nutrient transporters at the lactating mammary gland may be a mechanism to modulate the proper ontogeny of the neonatal system.

5.4.2. Increased Temporal Expression in Early Lactation

Nutrient transporters that increase transiently during early lactation and decline thereafter are members from the organic cation transporter family (Oct1), organic cation/carnitine transporter family (Octn2), nucleoside transporter family (Ent1, Cnt2, Cnt3), oligopeptide transporter family (Pept2) and the methotrexate carrier (Mtx1). The transient increase in these transporters may imply their role to actively accumulate critical compounds, which are main players in development (i.e. carnitine, nucleosides, peptides, and folate), into the breast milk during very early development to ensure sufficient levels are received by the nursing neonate. Levels declining thereafter may indicate a possible regulatory role (similar to that seen in the ontogeny of Dmt1[50, 51]) in modulating the ontogeny of the neonatal system. For instance, the mRNA levels of both Oct1 and Octn2 in the kidney (the site of highest expression[202]) increase postnatally, with levels initially very low and increasing gradually throughout suckling[202]. Similarly, mRNA levels and protein levels of Pept2 in the developing kidney show a similar pattern of development but at a much quicker rate[282], possibly reflecting the rapid changes in mammary gland mRNA levels. On the other hand, the intestinal development of Octn2 does not conform to this relationship. Octn2 mRNA and activity levels in the jejunum and ileum are significantly higher than adult values at birth and they decrease postnatally throughout the lactation period[283], where neither elimination of suckling, nor exogenous carnitine supplementation could alter the developmental expression pattern[283]. The adult levels of Octn2 increase distally in the small intestine[202], and the ontogeny of Octn2 at the duodenum is not known. Distal intestine regulation of Octn2 does not support the possible ontogeny-signaling mechanism of lactation, which is largely supported by the renal ontogeny of Octn2, Oct1, and Pept2, however we must consider the possibility of other transporters influencing the ontogeny by providing a nutrient stimulus (i.e. Octn3 ensuring proper carnitine levels as its expression continues to increase). However, the ontogeny of the remaining transporters has yet to be investigated. In addition, nutrients such as carnitine are conditionally essential and with time, the neonate develops the capacity to synthesize its own endogenous stores[229, 230]. Therefore, even though the ontogeny of carnitine transporters to not correspond to the mammary gland expression patterns, the availability of carnitine

in the milk will ultimately play a role in the ontogeny of carnitine synthesis in the neonate.

5.4.3. Increased Temporal Expression Throughout Lactation

Nutrient transporters that increase as lactation progresses are Cnt1 and Octn3. The ontogeny of these transporters is not known in other tissues and since their roles within the mammary gland are purely speculatory, their role in neonatal development is unknown. These transporters may contribute to milk composition of critical nutrients and play an important role in neonatal development. Furthermore, they may play a pivotal role in maintaining proper mammary gland functions in the later stages of lactation.

5.4.4. Constant Temporal Expression Throughout Lactation

The nutrient transporters that remain relatively constant throughout lactation are members of the organic cation/carnitine transporter family (Octn1), the equilibrative nucleoside transporter family (Ent2 & Ent3), the nucleobase transporter family (Ncbt1 & Ncbt2) and the oligopeptide transporter family (Pept1). The majority of these transporters are facilitators (Octn1[88], Ent2 & Ent3[86]) or low affinity transporters (Pept1[92]) and may only be expressed to maintain substrate flux across the mammary epithelial barrier. Interestingly, Octn1 mRNA levels in the kidney undergo a similar ontogeny as Octn2 and shows similar intestine expression patterns (increase distally) yet is expressed at much lower levels[202]. In addition, Octn1 levels are ubiquitously expressed at more consistent levels than the Octn2 transporter[202]. The role that Octn1 in the mammary gland may play in determining the proper ontogeny in the neonate is unknown. Conversely, the ontogeny of Pept1 mRNA in the neonatal intestine, and protein levels in the kidney have been evaluated[282]. The mRNA and protein levels in all 3 parts of the small intestine increases postnatally and spikes around day 5 of lactation and decreases thereafter to reach a lower, stable expression level[282] which does not support the potential relationship between Pept1 in the mammary gland modulating neonatal ontogeny. Therefore, the facilitated transporters may merely maintain substrate flux across the mammary epithelial cells and the ontogeny of these transporters may be predetermined.

5.5. Temporal Expression Patterns as Predictors of Transporter Importance & Nutritional Status in the Neonate

The temporal expression patterns in lactating mammary epithelial cells may play a significant role in determining the optimal composition of maternal milk, and in addition, may modulate the proper ontogeny of the neonatal system. If the latter proposition is correct, any manipulation of the nutrient transporters, either by maternal nutrition status or inhibition by exogenous compounds, will compromise the maternal composition of breast milk. This may ultimately result in an altered nutritional status leading to altered metabolic programming and ontogeny in the neonate. Furthermore, the temporal expression pattern may predict when a nutritional or exogenous modulation of transporter function is not only possible, but at which stage of lactation it may be extremely detrimental by interfering with important transporter functions. For instance, those transporters upregulated in early lactation imply a critical role in determining breast milk composition where any manipulation of transporter function may be detrimental.

5.5.1. Xenobiotics as Modulators of Maternal Nutrition Status

The ability of exogenous xenobiotics to alter the nutrient composition of maternal milk through competitive or noncompetitive reversible interactions has not been investigated. However, I have previously discussed the shared substrate profile of nutrient transporters between nutrients and clinically relevant xenobiotics[155, 157-161]. If nutrient transporters have the ability to transport xenobiotics, then there certainly does exist the possibility of having a xenobiotic-nutrient transporter interaction that decreases the transport of a critical nutrient substrate, which may result in a micronutrient deficiency. Therefore, it is imperative that a system be developed to screen such interactions.

5.6. Primary Mammary Epithelial Organoid (MEO) Cell Culture System

MEO isolated from female Sprague-Dawley rats at day 1 and day 10 of lactation were cultured in an extracellular matrix-rich environment as previously described[185, 193]. This was done to address the question to what extent primary mammary alveolar epithelial cells maintain transporter expression levels at the mRNA level and the culture

conditions necessary to maintain expression levels to provide a possible model for *in vitro* transporter uptake studies. The mRNA expression levels decreased throughout the plating period for Mrp1, Cnt3, and β-casein in both early and mid-lactation stage MEO, and the expression pattern of Octn2, Ent1, and Pept2 increased transiently by day 4 of culture and decreased thereafter in both MEO preparations. Octn3 followed a far different expression pattern as it continued to increase until day 8 of culture in early stage MEO and remained more stable in later differentiated cells. All transporter expression changes occurred at a much smaller degree in the MEO isolated from day 10 of lactation with the exception of the milk-protein β-casein indicating that the ECM environment in culture may have had a large influence on gene expression. Previous reports have cultured isolated MEO from virgin animals, which undergo significant proliferation, differentiation, and ductal morphogenesis in a sandwich culture of ECM proteins (Engelbreth-Holm-Swarm matrix (EHS); Matrigel™) with appropriate hormonal culture medium[185-189]. No studies to date have used this culture system to evaluate MEO isolated from lactating rats. It may be possible that the hormone-rich medium induces appropriate changes in the EHS matrix composition, or induces appropriate changes in the cellular architecture of organoids isolated from virgin animals, however such information is not available. Regardless of the mechanism, lactating MEO in EHS matrix does not perform as well as virgin MEO using the exact same culture conditions as evidence by the lack of β-casein gene expression.

The ECM matrix composition of the mammary gland differs depending on the maternal reproductive state (i.e. pregnancy, lactation, involution, or quiescent), where the ECM proteins laminin, tenascin, fibronectin, and clusterin[223] all change between pregnancy, lactation, and involution. It may be possible that the changes in ECM protein levels also change throughout suckling and therefore an ECM created from the EHS sarcoma (i.e. Matrigel™) may not match the composition of mammary gland ECM. In fact, the mammary ECM matrix has been shown to have up to a 50-fold higher fibronectin to laminin ratio than EHS matrix, which may better resemble the ECM composition that the mammary epithelial ductal tree is embedded in *in vivo*[223]. Accordingly, MEO cultured using an ECM composition of equal parts control matrix (EHS) and mammary gland matrix showed more favorable morphology than those

cultured on EHS matrix alone indicating that mammary gland matrix may be a far better cell medium for the culture and maintenance of mammary organoids[223].

On the other hand, the extracellular matrix composition may not be the governing factor in the maintenance of gene expression *in vitro*. After examining the temporal gene expression through the duration of culture, it became apparent that some of the expression patterns *in vitro* followed a similar pattern of expression *in vivo* (Mrp1, Octn2, Octn3, Cnt3, & β-casein), particularly in the young differentiated cells (lactation day 1). The similarities in expression patterns regardless of the physiological space (i.e. *in vivo* or *in vitro*) may indicate a predetermined genetic program governing gene expression. In fact, this idea has previously been proposed for the mammary epithelial cells during lactation[284]. This "alveolar switch" implies that transcription factors (possibly encoding a cell-cycle protein; Elf5[284] or Notch[285]) involved in coordinating the proliferation of mammary gland stem cells also play a key role in determining cell fate decisions. Therefore, the regulation of stem cell fate to either a secretory or non-secretory cell may predispose the cell to certain regulatory patterns, including gene expression, and consequently the cells may coordinate gene expression nearly independent of exogenous influences (i.e. tissue processing & culture conditions).

Nevertheless, all transporters evaluated maintained an mRNA signal at reasonable levels by day 4 of culture, which was reflected in the microscopic appearance of MEO, as they appeared to maintain cellular architecture up until day 4 of culture (Figure 4.15). Therefore, the cell culture conditions may need some modifications to create a more relevant cell culture system, as they currently do not accurately reflect *in vivo* mammary gland conditions. It would be beneficial to find a culture condition where transporter mRNA was maintained at a constant level for longer culture durations to facilitate the practicality of the methodology and the ability to extrapolate results as it is used to screen potential xenobiotic-transporter interactions *in vitro* as observed in other primary isolated cell systems. However, the current cell culture system does allow for the maintenance of transporter mRNA expression. This is consistent with the hypothesis that an ECM-rich culture environment will maintain transporter mRNA expression, at least at reasonable levels. Therefore, isolated MEO cultured in an ECM-rich environment for a short culture duration period has the

potential to serve as a rapid screening tool if used immediately post-isolation. More investigation is required to develop culture conditions that allow for prolonged culture durations and maintenance of transporter expression levels.

5.7. Limitations & Pitfalls

Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) is considered the benchmark technique for the evaluation of mRNA transcripts for its striking sensitivity[196, 286] and relatively low costs compared to microchip analyses[287, 288]. QRT-PCR is often done using two-reactions-two-tubes, or two-reactions-one-tube[196], and thus eliminates the need for "older" technologies that quantify transcripts using electrophoresis (i.e. northern blotting). However, despite the sensitivity, occasionally there is the need to pool tissue samples due to limited tissue availability or limited mRNA availability from selected tissue sources; the latter plagued this research. Therefore, to maximize resources, I had to pool samples at the MEO stage and extract total RNA thereafter. The method of "pooling" is not new to gene analysis, and is common-place when using more expensive analysis techniques such as microchip assays (recently reviewed[288, 289]). These reviews provide great insight into the effectiveness of appropriately pooled samples, and when pooled appropriately, allow for adequate statistical power (in addition to the marked cost reductions)[288, 289]. Recently reviewed by Kendziorski *et al*. (2003)[288], the technique of pooling was assessed using RT-PCR techniques. Using the more sensitive QRT-PCR analysis method, pooled samples from individuals did not introduce bias and resulted in averages that were extremely close to the means of individual analyses, with far less variation[288]. However, the variations in such pooled analyses are considered only to represent experimental variability and do not truly represent biological variation. Nevertheless, the findings indicate that using pooled mRNA samples with RT-PCR, one can obtain accurate results, representative of the population average[288, 289]. Even though a pooled sample allows for such accuracy, improper pooling can tremendously favor outliers, due, at least in part to the possibility of unequal sample sizes prior to pooling. Despite an attempt to eliminate this confounder, the role it may have played cannot be ignored.

Further potential pitfalls exist in the relationship between relative mRNA levels compared with those of controls. A marked change in mRNA levels does not always correlate with a similar magnitude of change in transporter protein or transporter activity. Therefore, further studies are warranted to investigate the alterations in transporter protein levels and activity, in order to fully characterize the temporal dynamics of the transporters throughout lactation.

5.8. Conclusion

My thesis work evaluated the constitutive expression of 3 drug transporters and 17 nutrient transporting proteins (including the milk protein β-casein) in mammary epithelial organoids (MEO), and compared that expression with whole mammary gland tissue isolated from female Sprague-Dawley rats at various stages of pregnancy, lactation, and involution. The temporal mRNA expression patterns and levels of transporters differ between the two tissue preparations for most transporters evaluated, possibly due to a heterogeneous cell population in the whole mammary gland sections or due to the presence of milk or blood fluids that is not present in the isolated MEO samples. The magnitude of the role that viable, sloughed cells in milk fractions may play in contributing to whole tissue mRNA analysis is unknown. However, the presence of these cells will unquestionably contribute to the total RNA pool[254, 273, 278]. Similarly, blood fractions that remain in the mammary gland can also contribute to the total RNA pool[275], and without perfusing the mammary gland prior to extraction, these fluid fractions will consistently add to the RNA pool in whole tissue samples. Therefore, since the MEO preparations are void of any fluid and cellular contaminants[185] and better represent the physical barrier between the maternal system and the breast milk, the mRNA profiling of each transporter in the MEO may allow for a more accurate evaluation of transporter functions at the lactating mammary gland. Furthermore, the unique temporal expression patterns of transporters in MEO may provide better insight into the critical windows of nutrient delivery, and the mechanisms of the nutrient transport across the mammary epithelia for their accumulation in breast milk.

The decreased expression of the ABC-transporters (Mdr & Mrp) implies a regulatory role in limiting drug exposures to the neonate. In addition, the down-regulation also demonstrates that the mammary gland is highly resourceful, conserving cellular economy by dedicating itself to providing nutrient-rich secretions and wastes no expense on transporters that do not contribute to this pivotal role. The decreased expression of Dmt1, the only SLC-transporter to undergo this expression pattern, is paralleled by similar decreases in Dmt1 protein levels[50-52], activity levels[50-52] and consequently milk iron levels[54, 274]. The decline of this transporter at all 3 functional levels is reflected in the neonate as the expression of its own compliment of iron transporters increases post-natally[50, 51, 281], which may indicate that the mammary gland regulation of milk composition is a critical signaling mechanism to the neonate to upregulate its own compliment of transport proteins.

The increased expression of SLC-transporters in early lactation (Oct1, Octn2, Ent1, Cnt2, Cnt3, Pept2 & Mtx1) may imply their role to actively accumulate critical compounds that are responsible for ensuring proper development (i.e. carnitine, nucleosides, peptides, and folate) into the breast milk during very early developmental stages. The decrease in transporter expression thereafter may indicate a possible regulatory role (similar to that seen in the ontogeny of Dmt1[50, 51]) in modulating the ontogeny of the neonatal system. However, a similar ontogeny-relationship does not exist for some of these transporters (i.e. Octn2[283]). One must consider the possibility of other transporters influencing the ontogeny by providing adequate nutrient stimulus (i.e. Octn1 & Octn3 ensuring proper carnitine levels). Therefore, the increase in Cnt1 and Octn3 throughout lactation may fulfill this role by providing the breast milk with adequate levels of nucleosides and carnitine, respectively. Furthermore, they may also serve to maintain proper mammary gland functions and thereby play a dual role within the mammary epithelia.

The relatively constant expression of Octn1, Ent2, Ent3, Ncbt1, Ncbt2, and Pept1 may indicate their role to maintain substrate flux across the mammary gland epithelia. Certainly, their role in other tissues as facilitators (Octn1[88], Ent2 & Ent3[86]) or low-affinity transporters (Pept1[92]) supports this idea. In addition, the lack of agreement between mammary gland regulation and the ontogeny of these some of these

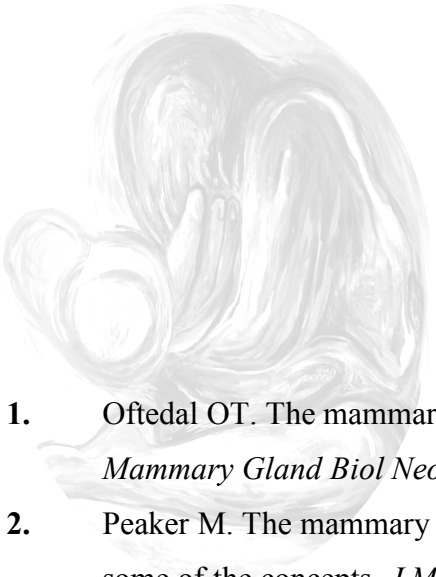
transporters (i.e. Octn1[202] & Pept1[282]) further supports the role that all members of a transporter family collectively, and not independently, influence ontogeny.

The complex regulation of transporters allows for the optimal nutrient composition of the breast milk, a composition that has the potential to influence the proper ontogeny of the neonatal system. Although the mammary gland limits xenobiotic exposure through the down-regulation of drug transporters and up-regulation of drug metabolizing enzymes, the threat remains that xenobiotics may occupy nutrient transporters and influence milk nutrient composition thereby leading to a reorganization of the neonatal system. Therefore, a reliable screening tool to evaluate this possibility would prove to be an invaluable resource for ensuring the health of the neonate. The current primary MEO cell culture system does not accurately reflect the *in vivo* mammary gland environment. However, it does allow for the maintenance of transporter expression at reasonable levels. If used immediately post-isolation, this system may provide a useful tool to evaluate the threat of a xenobiotic-transporter interaction at the lactating mammary gland, adding another dimension to ensuring neonatal health and well being.

Although this thesis work made a small but important contribution to our understanding of transporter expression in lactating mammary epithelial cells, many unanswered questions remain about transporter function and regulation throughout lactation.

5.9. Future Directions

mRNA expression profiling of various nutrient transporters throughout lactation is merely one small contribution to our understanding of mammary gland functions. Further investigations of the cellular/subcellular localization and activity characteristics of the mammary gland transporters are sorely needed to fully understand their roles in the transepithelial movement of nutrients into the breast milk. Furthermore, the xenobiotic substrate profile of these nutrient-based transporters will provide insight into the possibilities of xenobiotic-nutrient transporter inhibition. Screening for these interactions will add another assessment tool to help safeguard infant health by preserving the high quality composition of breast milk.



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